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Plasmodium falciparum Founder Populations in Western Cambodia Have Reduced Artemisinin Sensitivity *In Vitro*

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Reduced *Plasmodium falciparum* sensitivity to short-course artemisinin (ART) monotherapy manifests as a long parasite clearance half-life. We recently defined three parasite founder populations with long half-lives in Pursat, western Cambodia, where reduced ART sensitivity is prevalent. Using the ring-stage survival assay, we show that these founder populations have reduced ART sensitivity *in vitro* at the early ring stage of parasite development and that a genetically admixed population contains subsets of parasites with normal or reduced ART sensitivity.

Plasmodium falciparum resistance to frontline antimalarial drugs has repeatedly emerged in Southeast Asia and spread to Africa (1), prompting the World Health Organization in 2005 to recommend the worldwide use of artemisinin (ART)-based combination therapies (ACTs) for uncomplicated falciparum malaria (2). Parasites with reduced ART sensitivity have since become entrenched in western Cambodia (3–7), have emerged elsewhere in Southeast Asia (8–11), and are threatening the efficacy of all ACTs (12). Reduced ART sensitivity manifests as a long parasite clearance half-life in patients treated with an ART derivative or an ACT (13, 14). Based on a population structure analysis of genome-wide single-nucleotide polymorphism (SNP) data from 293 Cambodian parasites, we previously identified three highly differentiated founder populations (KH2, KH3, and KH4) of slow-clearing parasites in Pursat Province in western Cambodia and a subpopulation (KH1) of fast-clearing parasites in Ratanakiri Province in eastern Cambodia (15).

While the half-life distributions of KH2, KH3, and KH4 were similar (15), these data may be confounded by host factors (e.g., hemoglobin E [5], acquired immunity [16], and pharmacokinetics) or parasite stage at the time of ART treatment (17, 18), all of which may influence parasite clearance kinetics *in vivo*. To investigate the intrinsic ART sensitivity of founder populations in the absence of potential confounders, we sought to characterize them *in vitro* using the ring-stage survival assay ($RSA_{0-3\ h}$) (17). This assay measures the percentage of early (0- to 3-h) ring forms that survive a pharmacologically relevant dose (700 nM for 6 h) of dihydroartemisinin, the active metabolite of all ARTs. We selected 51 parasite isolates from Pursat and Ratanakiri (5, 15), adapted them to *in vitro* culture for several weeks, and genotyped them at 12 SNPs, as described previously (17). Four parasites that did not adapt to culture and three that differed genetically from the initial isolates were discarded.

The remaining 44 parasites (39 from Pursat, 5 from Ratanakiri) were genotyped from deep-sequencing read data at 681,546 high-quality exonic SNPs as part of the MalariaGEN *Plasmodium falciparum* community project (version 3.1 data release) (19). Based on an updated population structure analysis of genome-wide SNP data from 515 Cambodian parasites (O. Miotto et al., submitted for publication), we reassigned all 44 isolates to a core subpopulation (KH-C, $n = 6$), one of three western Cambodian founder populations (WKH-F01, $n = 5$; WKH-F02, $n = 3$; WKH-F04, $n = 11$), or an unclassified subpopulation (KH-U, $n = 19$). Using parasite clearance half-lives as phenotypes, KH-C was categorized as fast clearing, and WKH-F01, WKH-F02, and WKH-F04 were categorized as slow clearing (O. Miotto et al., submitted for publication). KH-U, which appears genetically admixed, shows a wide range of half-life values and cannot be reliably classified as fast clearing or slow clearing.

The median (range) half-life for parasites in KH-C was 2.68 h (1.58 to 4.84 h) and was significantly longer for parasites in WKH-F01 (7.18 h [4.67 to 8.21 h]; $P = 0.009$, Mann-Whitney test) (GraphPad Prism 6, GraphPad Software, La Jolla, CA), WKH-F02 (6.72 h [6.00 to 6.87 h]; $P = 0.024$), and WKH-F04 (6.32 h [4.49 to 8.54 h]; $P = 0.0006$) (Fig. 1A). Half-life values did not differ between the founder populations ($P = 0.556$, Kruskal-Wallis test). The distribution of half-lives in KH-U (median, 6.44 h [range, 3.31 to 10.1 h]) suggested that this group contains a mixture of fast-clearing and slow-clearing parasites (Fig. 1A). To investigate whether founder populations differ in their level of reduced ART sensitivity *in vitro* and whether KH-U parasites could be separated into parasites with normal or reduced ART sensitivity, we performed the $RSA_{0-3\ h}$ on all of the isolates.

The median (range) percent survival for parasites in KH-C was 0.22% (0.12% to 0.75%) and was significantly higher for parasites in WKH-F01 (30.0% [2.30% to 90.0%]; $P = 0.004$, Mann-Whitney test), WKH-F02 (82.6% [43.3% to 108%]; $P = 0.024$), and

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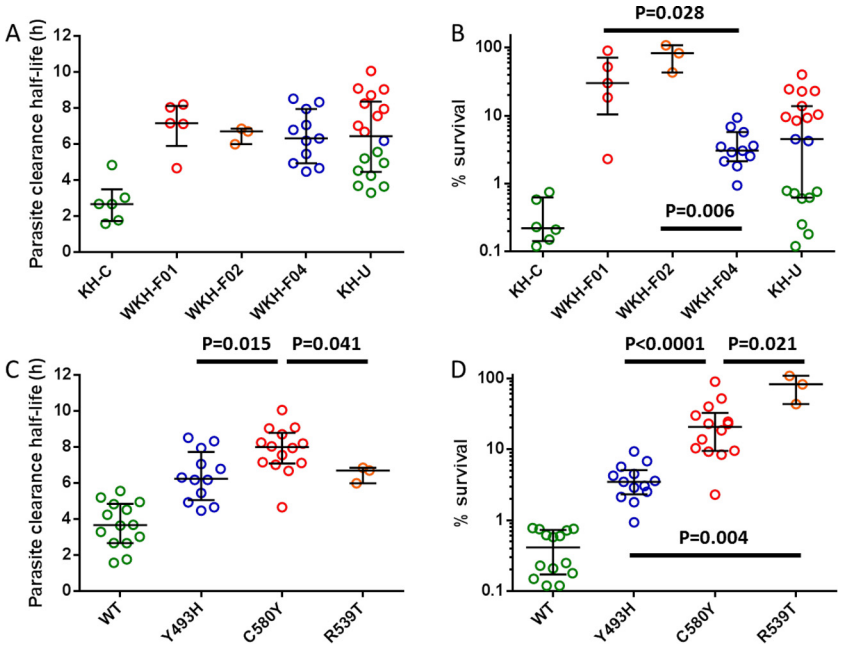

FIG 1 Half-lives and percent-survival values of Cambodian *Plasmodium falciparum* isolates stratified by parasite subpopulation (A and B) or K13-propeller allele (C and D). The color codes for the WT, Y493H, C580Y, and R539T alleles used in panels A and B are indicated in panels C and D. All founder and mutant populations had significantly longer half-lives and higher percent-survival values than core and WT populations. *P* values were calculated using the Mann-Whitney test and are shown for significant differences among the founder or mutant populations.

WKH-F04 (3.04% [0.94% to 9.36%]; $P = 0.0002$) (Fig. 1B). The percent-survival distributions differed among the founder populations ($P = 0.002$, Kruskal-Wallis test), with significantly higher values in WKH-F01 and WKH-F02 than in WKH-F04. These data show that all three founder populations have reduced ART sensitivities *in vitro* and that the percent-survival phenotype can vary between and within the founder populations. The median (range) percent survival for KH-U was 4.50% (0.12% to 40.0%), and the distribution of percent-survival values clearly shows that KH-U contains a mixture of parasites with normal or reduced ART sensitivity (Fig. 1B). Using data from all of the isolates, the percent survival values and half-lives correlated significantly ($r = 0.6965$, $P < 0.0001$, Spearman correlation test).

In Cambodia, mutations in the K13-propeller domain of a kelch protein (PF3D7_1343700) were recently associated with reduced ART sensitivity *in vivo* and *in vitro* (7). We investigated whether K13-propeller mutations are associated with founder populations and whether they distinguish parasites with normal or reduced ART sensitivity in KH-U. We found that the WKH-F01, WKH-F02, and WKH-F04 parasites harbored exclusively the C580Y, R539T, and Y493H alleles, respectively, while the KH-C parasites carried the wild-type (WT) allele (Fig. 1B). Within KH-U, the WT allele clearly identified parasites with normal ART sensitivity as having a survival rate of <1%, and the C580Y and Y493H alleles identified parasites with reduced ART sensitivity as having a survival rate of ≥1%. These data indicate that K13-propeller mutations are associated with specific founder populations and clearly segregate parasites with normal or reduced ART sensitivity in KH-U.

To explore whether K13-propeller mutations confer different levels of reduced ART sensitivity *in vivo* and *in vitro*, we stratified our data by K13-propeller alleles. The median (range) half-life for WT parasites was 3.67 h (1.58 to 5.58 h, $n = 14$) and was significantly longer for Y493H (6.26 h [4.49 to 8.54 h], $n = 14$; $P < 0.0001$, Mann-Whitney test), C580Y (8.01 h [4.67 to 10.1 h], $n = 14$; $P < 0.0001$), and R539T (6.72 h [6.00 to 6.87 h], $n = 3$; $P = 0.003$) parasites (Fig. 1C). The half-life values differed between the mutant populations ($P = 0.024$, Kruskal-Wallis test) and were significantly higher for C580Y parasites than for Y493H and R539T parasites. The median (range) percent survival for WT parasites was 0.41% (0.12% to 0.78%) and was significantly higher for Y493H (3.48% [0.94% to 9.36%]; $P < 0.0001$, Mann-Whitney test), C580Y (20.7% [2.30% to 90.0%]; $P < 0.0001$), and R539T (82.6% [43.3% to 108%]; $P = 0.003$) parasites (Fig. 1D). C580Y and R539T parasites had significantly higher percent-survival values than Y493H parasites, and R539T parasites had higher percent-survival values than C580Y parasites. These data indicate that parasites carrying the same K13-propeller mutation, even those within the same founder population, can differ substantially in their half-lives and percent-survival values, suggesting that genetic or other factors modulate the level of reduced ART sensitivity *in vivo* and *in vitro*. The possibility that R539T parasites, which have the highest percent-survival values, clear faster than C580Y parasites may indicate additional mutation-specific effects on parasite clearance or greater loss of fitness *in vivo*, but this requires further investigation.

In summary, the WKH-F01, WKH-F02, and WKH-F04 founder populations in Pursat have reduced ART sensitivity *in vitro* and are associated with the C580Y, R539T, and Y493H K13-propeller alleles, respectively. These founder populations and K13-propeller mutations may be associated with different levels of reduced ART sensitivity *in vivo* and *in vitro*. Compared to the parasite clearance half-life, the percent-survival phenotype more clearly discriminates parasites with normal or reduced ART sensitivity in KH-U. This is likely be-

cause the *in vitro* $RSA_{0-3\ h}$ measures the intrinsic susceptibility of stage-synchronized parasites to ART in the absence of host factors.

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