HHS Public Access

Author manuscript

Science. Author manuscript; available in PMC 2015 June 23.

Published in final edited form as:

Science. 2015 January 23; 347(6220): 428–431. doi:10.1126/science.1260867.

K13-propeller mutations confer artemisinin resistance in *Plasmodium falciparum* clinical isolates

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Abstract

The emergence of artemisinin resistance in Southeast Asia imperils efforts to reduce the global malaria burden. We genetically modified the *Plasmodium falciparum K13* locus using zinc-finger nucleases and measured ring-stage survival rates after drug exposure in vitro; these rates correlate with parasite clearance half-lives in artemisinin-treated patients. With isolates from Cambodia, where resistance first emerged, survival rates decreased from 13 to 49% to 0.3 to 2.4% after the removal of K13 mutations. Conversely, survival rates in wild-type parasites increased from ≤0.6% to 2 to 29% after the insertion of K13 mutations. These mutations conferred elevated resistance to

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All other authors declare no competing financial interests.

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recent Cambodian isolates compared with that of reference lines, suggesting a contemporary contribution of additional genetic factors. Our data provide a conclusive rationale for worldwide K13-propeller sequencing to identify and eliminate artemisinin-resistant parasites.

The worldwide use of artemisinin (ART)–based combination therapies (ACTs) for the treatment of *Plasmodium falciparum* malaria is the foundation of renewed efforts to eradicate this leading cause of childhood mortality (1, 2). The pharmacodynamic properties of clinically used ART derivatives [artesunate, artemether, and dihydroartemisinin (DHA)] can reduce the biomass of drug-sensitive parasites by four orders of magnitude every 48 hours (3), corresponding to a single cycle of asexual blood-stage *P. falciparum* development. The short half-life (typically <1 hour) of ART derivatives in plasma necessitates the use of longer-lasting partner drugs that can eliminate residual parasites once the ART component has dropped to subtherapeutic concentrations (4). The use of ACTs in expanded malaria control and elimination programs has yielded notable successes in recent years, contributing to an estimated 30% reduction in global mortality rates in the past decade (5).

These impressive gains, however, are now threatened by the emergence of ART resistance, first detected in western Cambodia and now observed in Thailand, Vietnam, and Myanmar (6, 7). The severity of this situation is underscored by the fact that resistance to piperaquine, an ACT partner drug, is emerging in western Cambodia (8, 9). No alternative, fully effective first-line therapy is currently available to replace ACTs, should ART fail globally. Clinically, ART resistance is defined as a long parasite clearance half-life (the time it takes for the peripheral blood parasite density to decrease by 50%) after treatment with ART monotherapy or an ACT (6, 10, 11). This metric correlates with the percentage of early “ring-stage” parasites (0 to 3 hours after invasion of human erythrocytes) that survive a pharmacologically relevant exposure to DHA (the active metabolite of all ARTs), as measured in the in vitro Ring-stage Survival Assay ($RSA_{0–3h}$) (12).

Recently, mutations in the propeller domain of the *K13* gene were identified as candidate molecular markers of ART resistance (13). This gene resides on chromosome 13 of the *P. falciparum* genome, near regions earlier associated with slow parasite clearance rates (14–16). K13 belongs to the kelch superfamily of proteins, whose propeller domain harbors multiple protein-protein interaction sites and mediates diverse cellular functions, including ubiquitin-regulated protein degradation and oxidative stress responses (17). The K13 M476I mutation was first observed in Tanzanian F32 parasites that were exposed in vitro to escalating concentrations of ART over 5 years, yielding the F32-ART line (13, 18). [Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. In the mutants, other amino acids were substituted at certain locations; for example, M476I indicates that methionine at position 476 was replaced by isoleucine.] Subsequent genomic analysis of Cambodian isolates identified four prevalent K13-propeller mutations (Y493H, R539T, I543T, and C580Y) that were associated with elevated $RSA_{0–3h}$ survival rates in vitro and long parasite clearance half-lives (>5 hours) in patients (13, 19). Determining whether K13-propeller mutations confer

ART resistance in clinical isolates and assessing the contributions of individual polymorphisms in distinct genetic backgrounds is essential to defining the underlying molecular mechanisms.

We developed zinc-finger nucleases (ZFNs) (20) to enable targeted genetic engineering of *K13* in newly culture-adapted Cambodian isolates and older established reference lines of *P. falciparum* (tables S1 and S2). ZFNs were introduced into cultured intra-erythrocytic parasites via electroporation with plasmids containing *K13* donor templates. ZFNs triggered double-stranded breaks in the *K13* genomic target locus of this haploid organism, leading to DNA resection and repair events that captured mutations delivered by pZFN^{K13}-hdhfr plasmids (fig. S1). Donor plasmids contained additional synonymous mutations that preclude ZFN binding while preserving the K13-translated amino acid sequence across that same stretch of DNA base pairs. These silent ZFN binding-site mutations protected the donor sequence and prevented the edited recombinant locus from being recleaved by the nucleases. Plasmids contained either the wild-type *K13* allele or one of several mutations (present in the six-blade K13-propeller domain) found in ART-resistant Cambodian isolates or F32-ART. This strategy successfully introduced or removed mutations in a set of *P. falciparum* clinical isolates from Cambodia, the epicenter of emerging ART resistance, as well as reference laboratory lines from distinct geographic origins (Fig. 1 and table S3). Of note, $RSA_{0–3h}$ assays comparing parental and edited control parasites showed no difference if only the binding-site mutations were introduced into the K13-propeller domain, indicating that these synonymous mutations were phenotypically silent (Fig. 1 and fig. S2). Independent assays with the same parasite lines tested by our different groups yielded consistent survival rates between laboratories (fig. S3).

Using donor plasmids containing a wild-type *K13*-propeller sequence and silent binding-site mutations, we generated a series of clones in which individual K13 mutations were removed from ART-resistant Cambodian isolates. One of these isolates (Cam3.II) showed slow clearance after ART monotherapy (in vivo half-life 6.0 hours) (table S2). Parental Cam3.I^{R539T} and Cam3.II^{R539T} isolates harboring the R539T mutation showed 40 to 49% $RSA_{0–3h}$ survival, whereas edited Cam3.I^{rev} and Cam3.II^{rev} clones carrying the reverted wild-type allele showed only 0.3 to 0.7% survival (Fig. 2, A and B, and table S4). These highly significant differences in the survival rates of ring-stage parasites exposed to elevated DHA concentrations confirm the importance of R539T in mediating in vitro ART resistance in Cambodian isolates. Significant reductions in $RSA_{0–3h}$ survival rates were also observed upon removal of I543T (43% in Cam5^{I543T} versus 0.3% in Cam5^{rev}) (Fig. 2C) and C580Y (13% in Cam2^{C580Y} versus 2.4% in Cam2^{rev}) (Fig. 2D).

We also assessed the impact of introducing K13 mutations into a fast-clearing Cambodian isolate (CamWT; in vivo half-life 3.7 hours) (table S2), the Cam3.II^{rev} clone, and three reference lines (V1/S, F32-TEM, and FCB). CamWT and Cam3.II^{rev} parasites harboring wild-type *K13* alleles showed 0.6 to 0.7% $RSA_{0–3h}$ survival, whereas the corresponding C580Y-edited clones yielded 9 and 24% survival, respectively (Fig. 2, E and F). Introducing R539T into V1/S caused a similar increase in $RSA_{0–3h}$ survival (0.3 to 21%) (Fig. 2G and table S4). Editing F32-TEM to express M476I caused a moderate increase in $RSA_{0–3h}$ survival (<0.2% in F32-TEM to 1.7% in F32-TEM^{M476I}) (Fig. 2H). We also observed

modest in vitro resistance in FCB parasites edited to express C580Y, with $RSA_{0–3h}$ survival increasing from 0.3% in the parental line to 1.9% in FCB^{C580Y} parasites (Fig. 2I). This result differs from a recent study of the use of Cas9 in *P. falciparum*, which reported a greater increase in $RSA_{0–3h}$ survival (11 to 15%) in two clones engineered to express K13 C580Y (21). That report used the drug-sensitive NF54 strain—which was isolated decades before ART use and the emergence of resistance (22)—and did not examine additional mutations or assess the impact of removing K13 mutations from ART-resistant clinical isolates.

In contrast to the substantial changes we observed in the $RSA_{0–3h}$, standard in vitro dose-response measurements by use of parental and *K13*-edited V1/S and Cam3.II parasites revealed no effect of R539T or C580Y on DHA or artesunate median inhibitory concentration (IC_{50}) values (fig. S4). This finding is consistent with earlier studies that showed no correlation between IC_{50} values and clinical ART resistance (6, 10, 12).

We subsequently investigated whether individual mutations confer different levels of ART resistance in the $RSA_{0–3h}$. In the Dd2 reference line, the introduction of M476I, R539T, or I543T mutations conferred considerably higher degrees of resistance than those of Y493H and C580Y (10 to 30% versus 2 to 4% survival, respectively) (Fig. 2J and table S4). These data corroborate the recent observation of higher levels of in vitro resistance in Cambodian isolates containing the R539T mutation as compared with Y493H or C580Y (23).

The relatively modest increase in survival of C580Y-expressing Dd2 parasites compared with R539T- and I543T-expressing clinical isolates and edited clones was quite unexpected, given that C580Y has rapidly become the predominant mutant allele in western Cambodia (7, 13). We thus explored the impact of C580Y in different genetic backgrounds. Introducing C580Y conferred greater levels of resistance in three Cambodian isolates as compared with Dd2 and FCB parasites (Fig. 2K), suggesting a role for additional parasite factors in augmenting K13-mediated resistance in these contemporary field isolates. The disparity between relatively low in vitro resistance conferred by C580Y and its widespread dissemination in Cambodia might be explained by a lower fitness cost or increased transmission potential of C580Y-expressing parasites, or by the parasite genetic background.

Cambodian parasites are specifically characterized by sympatric subpopulations that show only limited genetic admixture and that generally harbor distinct K13 mutations (16). These findings suggest that K13 mutations might have arisen preferentially on backgrounds with favorable genetic factors. In this context, recent comprehensive analyses of K13 mutations across multiple sites in Southeast Asia have documented a series of additional mutations associated with slow clearance rates in Cambodia, Thailand, Myanmar, Laos, and Vietnam (7, 24). K13 mutations have also been observed in African isolates (7, 25, 26), although none of these correspond to the most prevalent mutations in Cambodia, and ART or ACT treatments in African sites continue to show a high level of efficacy (7). A recent deep-sequencing study of the K13-propeller domain in more than 1110 *P. falciparum* infections collected from 14 sites across sub-Saharan Africa identified a large reservoir of naturally occurring K13-propeller variation, whose impact on artemisinin susceptibility is unknown and requires further investigation. These polymorphisms include one rare mutation

previously observed in Cambodia (P553L) and several others (including A578S) close to known resistance-causing mutations in the propeller domain (26). Our gene-editing system can now be used to comprehensively dissect K13 polymorphisms across malaria-endemic regions and identify those that confer ring-stage ART resistance.

Mode-of-action studies have shown that ARTs are active against all asexual blood stages of parasite development. In the more mature trophozoite stages, ARTs are activated after hemoglobin degradation and liberation of reactive heme whose iron moiety can cleave the endoperoxide linkage of these sesquiterpene lactone drugs (27). Activation generates free radicals that are thought to trigger oxidative stress and damage cellular macromolecules, including parasite membrane components, proteins, and neutral lipids (28, 29). Recent evidence suggests that hemoglobin degradation begins early after merozoite invasion, potentially providing a source of ART activator in ring-stage parasites (30). Our $RSA_{0–3h}$ data support earlier evidence that reduced ring-stage susceptibility accounts for the clinical phenotype of slow parasite clearance after ART treatment (12, 31). K13 mutations might achieve this by protecting parasites from the lethal effects of ART-induced oxidative damage, potentially via a cellular pathway similar to antioxidant transcriptional responses regulated by the mammalian ortholog Keap1 (32). Our set of K13-modified isogenic parasites with different levels of ART resistance on distinct genetic backgrounds now enables a search for K13-interacting partners and delivers tools to interrogate the underlying mechanism.

Our data demonstrate a central, causal role for K13-propeller mutations in conferring ART resistance in vitro and provide a molecular explanation for slow parasite clearance rates in patients (6, 7, 10). By exposing greater parasite biomasses to ACTs in vivo, K13-propeller mutations may promote the evolution of partner drug resistance (8, 9) and higher-grade ART resistance. Our study thus offers a conclusive rationale for a global K13 sequencing effort to track the spread of ART resistance and mitigate its impact on malaria treatment and control programs, particularly in hyperendemic regions in Africa.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

D.A.F. gratefully acknowledges funding from the NIH (R01 AI109023). This study was supported in part by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, NIH, the French “Agence Nationale de la Recherche” (ANR-13-BSV3-0018-01 and the Laboratoire d’Excellence IBEID), and the Institut Pasteur, Division International (ACIP A-10-2010). Parental and transgenic parasite lines have been deposited and are being made available through BEI Resources (www.mr4.org) with the following accession numbers: MRA-1240, Cam3.IR539T (also known as IPC 5202); MRA-1252, Cam3.Irev; MRA-1241, Cam5I543T (also known as IPC 4912); MRA-1253, Cam5rev; MRA-1236, Cam2C580Y (also known as IPC 3445); MRA-1254, Cam2rev; MRA-1250, CamWT; MRA-1251, CamWTC580Y; MRA-150, Dd2; MRA-1255, Dd2R539T. Parasite lines generated for this study will also be provided upon request from D.A.F. Requests for ZFNs should be directed to F.D.U. (FUrnov@sangamo.com); a materials transfer agreement is required. We extend our gratitude to F. Ariey (Institut Pasteur, Paris) for his important contribution to initiating this study, I. McKeague and O. Lieberman (Columbia University Medical Center) for their statistical and scientific input, and E. Rebar and the Production Group at Sangamo BioSciences for ZFN assembly and validation. L.Z., S.L., P.D.G., and F.D.U. declare that they are full-time employees of Sangamo, which designed, validated, and provided the ZFNs used in this study. B.W., O.M.-P., F.B.-V., and D.M., are co-inventors on the pending patents US61/904651 and US62/062439, and N.K. is a

co-inventor on the pending patent US62/062439. Both patents are filed by Institut Pasteur. These patents cover the use of K13 mutations as a molecular marker of *P. falciparum* ART resistance. Sangamo holds patents on engineered DNA-binding proteins and the use thereof in targeted genome engineering and gene-specific regulation.

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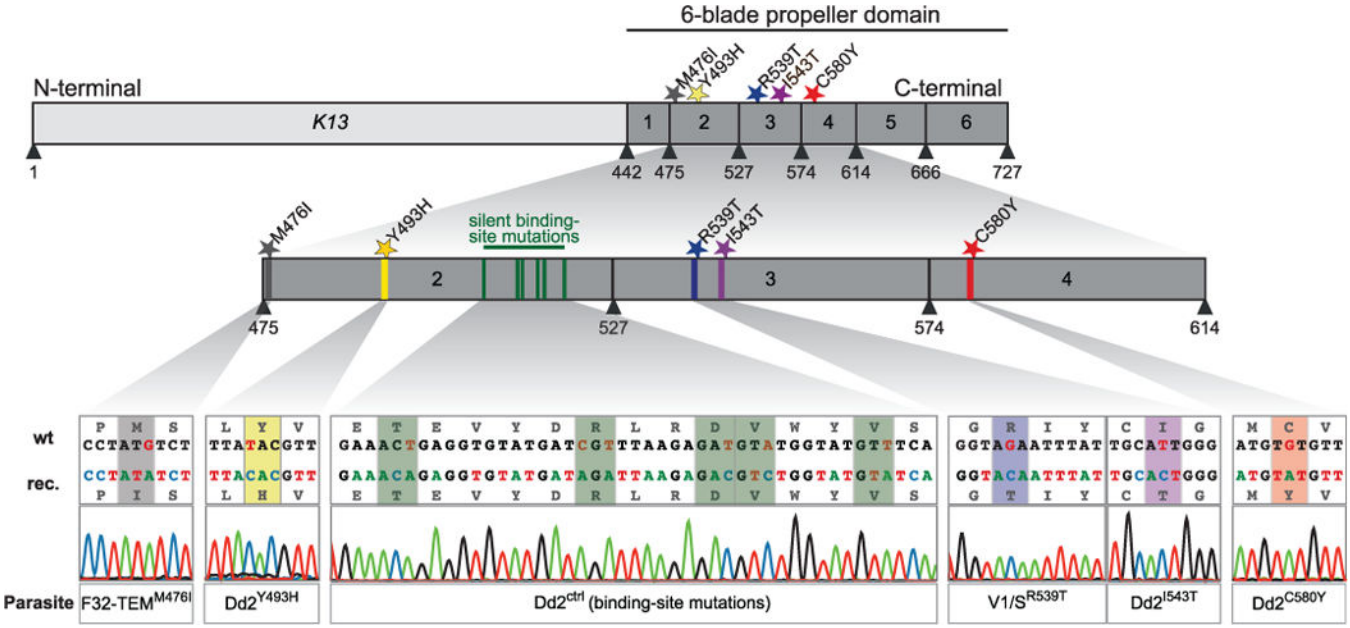

Fig. 1. Genetic modification of the K13-propeller domain

Location of K13-propeller mutations and sequencing results showing the insertion of individual mutations into recombinant parasites used in the $RSA_{0–3h}$. $Dd2^{ctrl}$ parasites contain only synonymous, phenotypically silent binding-site mutations and showed 0.7% survival rates, which is equivalent to those of parental Dd2 parasites (fig. S2).

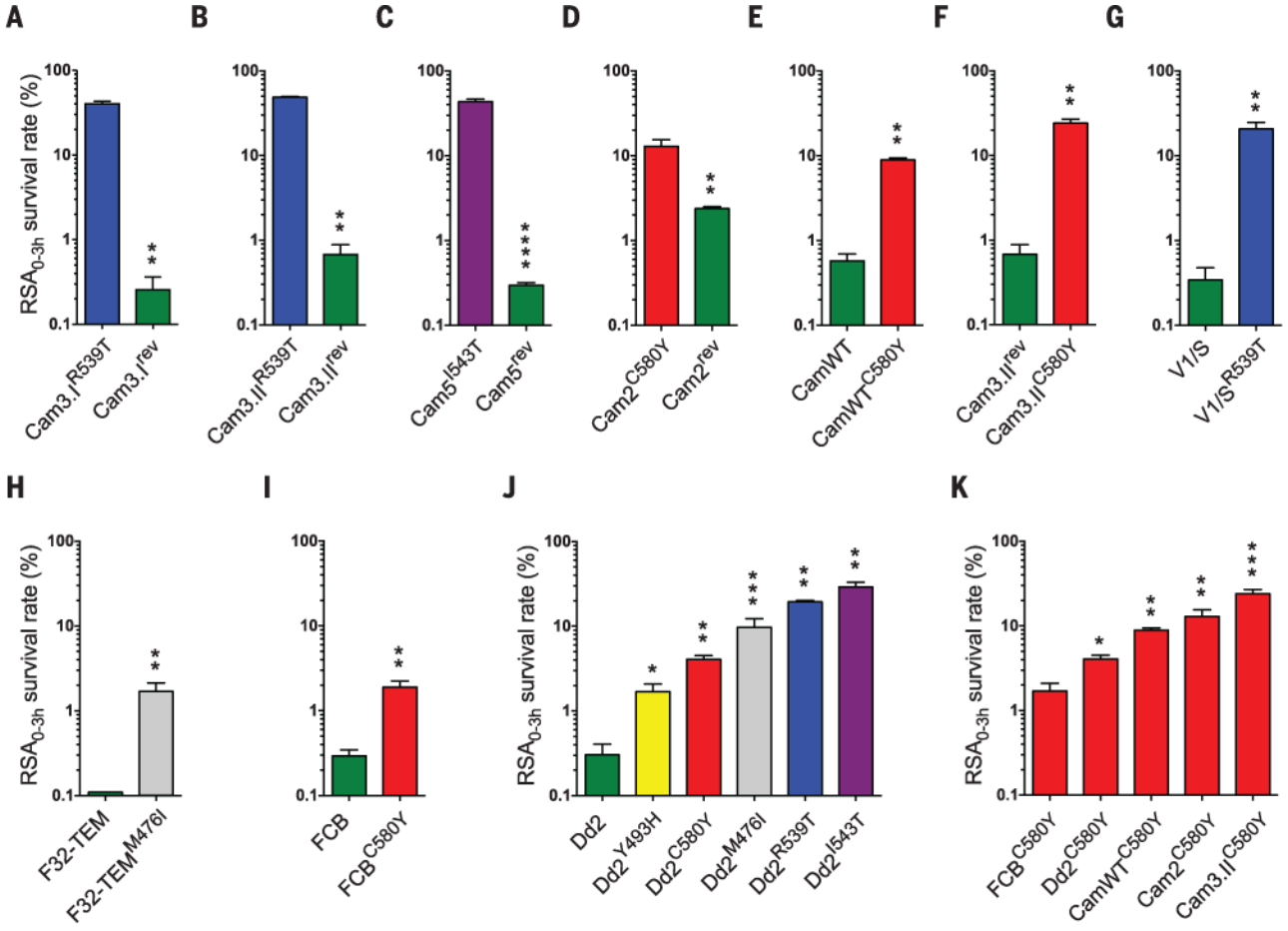

Fig. 2. K13-propeller mutations confer artemisinin resistance in clinical isolates and reference lines in vitro, as defined in the $RSA_{0–3h}$

Results show the percentage of early ring-stage parasites (0 to 3 hours after invasion of human erythrocytes) that survived a 6-hour pulse of 700 nM DHA (a pharmacologically relevant concentration of the active metabolite of ARTs), as measured by microscopy 66 hours later. Data show mean ± SEM percent survival compared with control dimethyl sulfoxide–treated parasites processed in parallel. (**A** to **D**) $RSA_{0–3h}$ survival for Cambodian isolates harboring native K13 mutations (shown in superscript) and ZFN-edited isogenic clones carrying wild-type *K13* alleles (superscript “rev”). (**E** to **I**) $RSA_{0–3h}$ survival for Cambodian isolates and reference lines harboring wild-type *K13* alleles and ZFN-edited isogenic clones carrying individual K13 mutations (shown in superscripts). (**J**) Impact of different K13 mutations on $RSA_{0–3h}$ survival in the Dd2 reference line, showing that I543Tand R539Tconfer the highest levels of resistance. (**K**) Introduction of C580Y into multiple Cambodian clinical isolates and reference lines, showing that this mutation confers varying degrees of in vitro resistance depending on the parasite genetic background. The geographic origins and known drug-resistance genotypes of these isolates and lines are provided in table S2. Results were obtained from 3 or 4 independent assays performed in duplicate (values provided in table S4; F32-TEM showed <0.2% $RSA_{0–3h}$ survival). Two-sample t tests with unequal variances (performed with the STATA package) were used to assess for statistically significant differences between *K13*-edited clones and their

comparator lines—the parental isolates listed on the left in (A) to (J) and the FCB^{C580Y} clone in (K) (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). Statistical outputs (including calculations of the SE of the difference between the means of samples being compared and the *P* values) are listed in the supplementary materials.