**Plasmodium falciparum** K13 Mutations Differentially Impact Ozonide Susceptibility and Parasite Fitness *In Vitro*

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**ABSTRACT** The emergence and spread in Southeast Asia of *Plasmodium falciparum* resistance to artemisinin (ART) derivatives, the cornerstone of first-line artemisinin-based combination therapies (ACTs), underscore the urgent need to identify suitable replacement drugs. Discovery and development efforts have identified a series of ozonides with attractive chemical and pharmacological properties that are being touted as suitable replacements. Partial resistance to ART, defined as delayed parasite clearance in malaria patients treated with an ART derivative or an ACT, has been associated with mutations in the *P. falciparum* K13 gene. In light of reports showing that ART derivatives and ozonides share similar modes of action, we have investigated whether parasites expressing mutant K13 are cross-resistant to the ozonides OZ439 (artefenomel) and OZ227 (arterolane). This work used a panel of culture-adapted clinical isolates from Cambodia that were genetically edited to express variant forms of K13. Phenotypic analyses employed ring-stage survival assays (*ring-stage survival assay from 0 to 3 h [RSA0–3h]*) whose results have earlier been shown to correlate with parasite clearance rates in patients. Our results document cross-resistance between OZ277 and dihydroartemisinin (DHA), a semisynthetic derivative of ART, in parasites carrying the K13 mutations C580Y, R539T, and I543T. For OZ439, we observed cross-resistance only for parasites that carried the rare K13 I543T mutation, with no evidence of cross-resistance afforded by the prevalent C580Y mutation. Mixed-culture competition experiments with isogenic lines carrying modified K13 revealed variable growth deficits depending on the K13 mutation and parasite strain and provide a rationale for the broad dissemination of the fitness-neutral K13 C580Y mutation throughout strains currently circulating in Southeast Asia.

**IMPORTANCE** ACTs have helped halve the malaria disease burden in recent years; however, emerging resistance to ART derivatives threatens to reverse this substantial progress. Resistance is driven primarily by mutations in the *P. falciparum* K13 gene. These mutations pose a threat to ozonides, touted as promising alternatives to ARTs that share a similar mode of action. We report that DHA was considerably more potent than OZ439 and OZ277 against ART-sensitive asexual blood-stage parasites cultured *in vitro*. We also document that mutant K13 significantly compromised the activity of the registered drug OZ277. In contrast, OZ439 remained effective against most parasite lines expressing mutant K13, with the exception of I543T that merits further monitoring in field-based OZ439 efficacy studies. K13 mutations differed considerably in their impact on parasite growth rates, in a strain-dependent context, with the most prevalent C580Y mutation being fitness neutral in recently culture-adapted strains from Cambodia, the epicenter of emerging ART resistance.
The substantial decline in malaria cases (22%) and deaths (50%) worldwide since 2000 can be largely attributed to the implementation of artemisinin (ART)-based combination therapies (ACTs), as first-line treatment in countries where malaria is endemic, and to expanded Anopheles mosquito vector control measures (1). However, the emergence and rapid spread of artemisinin-resistant strains of Plasmodium falciparum in the Greater Mekong subregion (historically the epicenter of antimalarial drug resistance) threaten ongoing efforts to eradicate this leading infectious cause of infant mortality. The situation could turn into a public health calamity if ART resistance manifests on the African continent, home to 90% of the malaria-related cases and deaths (2).

ART (a natural product from the Chinese sweet wormwood Artemisia annua) and its clinically employed semisynthetic derivatives, namely, dihydroartemisinin (DHA), artemether, and artesunate, are fast acting and potent antimalarial agents that can reduce the biomass of drug-sensitive asexual blood-stage parasites by up to 104-fold every 48 h (corresponding to one generation of the intraerythrocytic developmental cycle). A major limitation of these compounds is their very short half-life in plasma (<1 h), necessitating the addition of a longer-lasting partner drug to clear the infection (3). Emerging resistance to ART was first documented as prolonged parasite clearance times in P. falciparum-infected patients treated with artesunate in western Cambodia (4, 5). As a result, ACT partner drugs encounter larger numbers of parasites in ART-resistant infections, increasing the potential for partner drug resistance. Indeed, resistance to mefloquine and piperaquine has been documented in Cambodia, where it has been associated with high levels of ACT treatment failures, as measured by 28-day follow-up efficacy studies (6–9).

A genetic marker for ART resistance has been mapped to the P. falciparum K13 (Kelch13) locus by means of whole-genome sequencing of in vitro ART-pressured parasites as well as ART-resistant or -sensitive Cambodian clinical isolates (10). Multiple mutations have been found throughout K13, often situated within the six-bladed β-propeller domain of this protein (11–14). Gene editing experiments with several K13-propeller mutations, including R539T, I543T, and C580Y, have confirmed their causal role for in vitro ART resistance in Southeast Asian field isolates as well as in laboratory-adapted strains (15, 16).

Starting with the epicenter of K13-mediated ART resistance in western Cambodia, mutations have now spread to all neighboring countries and beyond, including Vietnam, Laos, Thailand, China, Myanmar, Bangladesh, and India (12, 17, 18). The most predominant mutation is C580Y, which has reached fixation in parts of Cambodia and near the Thailand-Myanmar border (9, 10, 13, 17, 19, 20). The prevalence of the C580Y mutation across all of Southeast Asia has been reported to be 26.5% to 48.3%, with some regions reporting a prevalence of up to 74% (12, 21, 22). The R539T mutation confers high levels of in vitro resistance and has been associated with delayed parasite clearance in patients; however, compared to C580Y, it is less prevalent, with 3.5% of K13 mutations in Cambodia-Vietnam-Lao People’s Democratic Republic (PDR) and 0.3% in Thailand-Myanmar-China (10, 12, 13, 15). Similarly, the I543T mutation has been associated with increased survival in vitro and a longer parasite clearance half-life in patients, but its prevalence was recently reported to reach only 2% in Southeast Asia (22). One potential explanation for the relatively high prevalence of the C580Y mutation, despite its lower degree of resistance observed in vitro, would be its enhanced fitness relative to other mutant isoforms. One measure of fitness is the rate of parasite asexual blood-stage growth (23), which we quantify herein using isogenic parasite lines that express mutant or wild-type K13.

Thus far, no fully effective alternative first-line treatment is available to replace ACTs, spurring efforts to find new potent antimalarial drugs (24, 25). Ideally, the new
molecules should not be affected by the current resistance mechanisms, should be able
to achieve full cure with a single-dose regimen, and should not depend on a natural
product whose supply levels can vary. ART, which possesses a 1,2,4-trioxane heterocy-
cle core, has inspired the development of structurally diverse fully synthetic peroxide
antimalarials, including the first synthetic ozonide OZ277 (arterolane maleate, RBx
11160) and the “next-generation” ozonide OZ439 (artefenomel) (26–28) (see Fig. S1 in
the supplemental material). Importantly, both ozonides exhibit longer half-lives in
plasma compared to the endoperoxide DHA. The half-life of OZ277 in plasma is only 2–
to 3-fold longer than for DHA, with a lower plasma exposure in
P. falciparum
-infected
patients compared to healthy volunteers (29, 30). OZ277 is nevertheless available in
India in combination with piperaquine (Synriam) (31). In contrast, OZ439 exhibits a
plasma half-life of 42 to 62 h and shows no reduced drug exposure in patients with
acute malaria (32, 33). This promising candidate for a single-dose oral cure, with a good
safety profile and low projected cost of goods, is currently undergoing clinical phase 2
trials in combination with ferroquine (ClinicalTrials.gov NCT02497612). OZ439 shows
very promising antimalarial efficacy (against both P. falciparum and Plasmodium vivax)
and excellent absorption, distribution, metabolism, and excretion properties (32).

ART and ozonides contain the same bioactive endoperoxide bridge (Fig. S1) and are
thought to be similarly activated via iron-catalyzed reductive scission of the endoper-
oxide bond, with parasite-digested hemoglobin providing a major source of iron
activator (34). One leading model is that this reaction generates free carbon-centered
radicals, which in turn alkylate parasite proteins as well as other biomolecules leading
to parasite killing (35–37). ART derivatives and ozonides both display very similar
activity profiles throughout the parasite life cycle, including activity against all stages of
asexual blood-stage development, including early rings (38, 39). Here, we examine
whether K13 mutations that confer ART resistance also mediate cross-resistance to
OZ277 and OZ439. These studies employ a series of culture-adapted isogenic Southeast
Asian parasite lines engineered to express mutant or wild-type K13 (15).

RESULTS AND DISCUSSION

DHA is a more potent inhibitor of parasites expressing wild-type K13 in vitro
compared to OZ439 and OZ277. In light of the structural similarities and presumed
similar modes of action between ozonides and DHA, we first examined their in vitro
parasite killing capacities against ART-sensitive parasites (Fig. 1). Using the ring-stage

FIG 1 DHA is a more potent inhibitor of parasite survival in vitro than OZ439 or OZ277, as defined in the
ring-stage survival assay from 0 to 3 h (RSA0–3h). Results show the percentage of early ring-stage parasites
expressing wild-type K13 (0 to 3 h postinvasion of human erythrocytes) that survive exposure to a 4-h
pulse of DHA, OZ439, or OZ277 ranging in concentration from 700 nM to 0.6 nM, as measured by flow
cytometry 68 h later. Data show mean ± standard error of the mean (SEM; error bars) percent survival
from at least three independent experiments performed in duplicate compared with DMSO vehicle-
treated parasites processed in parallel. Data show the combined results for percent survival in four
parasite lines harboring the wild-type K13 allele: CamWT, Cam3.II rev, V1/Y30M, and Cam5 rev. RSA0–3h
dose-response curves are shown for DHA, OZ439, and OZ277.
survival assay (ring-stage survival assay from 0 to 3 h [RSA0–3h]) that correlates with delayed parasite clearance (40), we assessed the susceptibility of 0- to 3-h ring stages of three recently culture-adapted Cambodian clinical isolates (Cam3.IIrev, CamWT, and Cam5rev [Cam stands for Cambodian, rev stands for revertant of the mutant allele back to its wild-type sequence, and WT stands for wild type]) as well as an older reference line from Vietnam (V1/S). Each of these P. falciparum lines expresses a wild-type K13 allele (see Table S1 in the supplemental material). The standard RSA0–3h determines the percentage of early ring-stage parasites that survive a single pharmacologically relevant dose of drug, i.e., 700 nM for DHA applied for 6 h. The rate of survival measured in vitro has been shown to correlate well with the lengthened parasite clearance half-lives observed in patients infected with ART-resistant malaria (40). With ART-resistant infections, these clearance half-lives (i.e., the time required to reduce the parasite biomass by half) are generally >5 h, in contrast to ART-sensitive infections that show half-lives closer to 2 to 3 h (41).

To obtain a comprehensive evaluation of the in vitro potency of OZ439 and OZ277, we modified the assay by exposing early ring-stage ART-sensitive parasites to a gradient of drug concentrations ranging from 700 nM to 0.6 nM for 4 h. Our protocol also employed multiple steps of washing and plate transfers to remove residual drug (see Materials and Methods), as a prior study reported that for OZ277 and more so for OZ439, the single washing step employed in the original RSA0–3h protocol failed to eliminate these ozonides and thus overestimated their activity (39) (a finding that we also observed). From these results, we determined the drug concentration required to inhibit 50% survival, defined as the RSA0–3h50%. This value was derived following nonlinear curve fitting of log-transformed data combined across our four parasite lines expressing the wild-type K13 gene, listed above. Results showed that DHA (3.7 nM) was more potent than OZ439 (17.2 nM) and OZ277 (33.1 nM) in its mean 50% inhibition of parasite survival (i.e., RSA0–3h50%), with the difference in efficacy being the most striking at concentrations below 45 nM (Fig. 1). As an example, only 5.1% of ring-stage ART-sensitive parasites (assayed at 0 to 3 h postinvasion) survived a 4-h pulse of 22 nM DHA, compared to 40.3% and 64.3% that survived a pulse with the same concentration of OZ439 and OZ277, respectively (Fig. 1).

The reduced parasite killing activities of OZ439 and OZ277 agree with the longer parasite clearance half-lives observed in ozonide-treated malaria patients (4 to 6 h and ~9 h for OZ439 and OZ277, respectively) compared to DHA (2 to 3 h) (32, 42). In our assays, OZ439 nonetheless showed complete inhibition of parasite growth when assayed at 700 nM for 4 h, a concentration just below the mean peak plasma concentration (721 nM) reported after a single dose of 200 mg of OZ439 in a recent patient cohort (32). With OZ277, complete inhibition of parasite survival was never observed, with the maximum pulse concentration of 700 nM affording a 3.6% survival. In comparison, peak plasma concentrations of 112 nM to 155 nM were observed in individuals receiving either a recommended 3-day regimen of 150 mg/day OZ277 in combination with piperaquine (750 mg/day) or a 7-day course of 150 mg daily of OZ277 monotherapy (29, 31, 42).

These results corroborate a recently published study, which reported that ozonides were less effective than DHA in terms of lowering rates of parasite survival when used in short pulses (3 to 9 h) (39). The basis for the reduced activity of ozonides in vitro is a matter of speculation. The possibility of a faster degradation of the ozonides can be excluded, since their stability has been found in blood-stage cultures to exceed the 4-h duration of our assays. In fact, DHA was found to be the least stable compound with a loss of 50% of its activity after 8 h, whereas the stability half-life of OZ277 was 17 h and OZ439 was stable well beyond 48 h, at which time it had lost only 20% of its activity (39). DHA and ozonides share a similar pharmacophore, and their modes of action are not expected to differ substantially. It is widely accepted that the antiparasitic activity of ozonides also involves the generation of carbon-centered radicals, which lead to oxidative stress and cell damage, as has been proposed for DHA (27, 43). One possible explanation for the different potencies could be related to slower activation of the
TABLE 1  Ring-stage survival assay percent survival values from drug-treated mutant-K13 or wild-type-K13 linesa

| Parasite          | 700 nM DHA (mean ± SEM) | P value | Fold change | 700 nM OZ439 (mean ± SEM) | P value | Fold change | 700 nM OZ277 (mean ± SEM) | P value | Fold change |
|-------------------|-------------------------|---------|-------------|---------------------------|---------|-------------|---------------------------|---------|-------------|
| Cam3.IIIrev       | 0.9 ± 0.3               | 3       |             | 1.1 ± 0.2                 | 3       |             | 9.0 ± 2.4                 | 3       |             |
| Cam3.IIC580Y      | 9.2 ± 2.7               | <0.05   | 10.5        | 1.4 ± 0.7                 | 3       | ns          | 22.3 ± 3.6                | 3       | ns          |
| Cam3.IIR539T      | 21.5 ± 1.9              | <0.05   | 24.5        | 1.9 ± 0.8                 | 3       | ns          | 29.8 ± 2.4                | 3       | <0.05       |
| V1/Srev           | 0.4 ± 0.1               | 3       |             | 0.7 ± 0.3                 | 3       |             | 2.5 ± 0.4                 | 3       |             |
| V1/C580Y          | 6.4 ± 0.5               | <0.05   | 15.0        | 0.5 ± 0.2                 | 3       | ns          | 9.4 ± 0.9                 | 3       | <0.01       |
| V1/SR539T         | 22.6 ± 3.6              | <0.01   | 53.1        | 2.9 ± 1.4                 | 4       | ns          | 24.3 ± 4.1                | 4       | <0.001      |
| Cam5I543T         | 0.5 ± 0.1               | 4       |             | 0.7 ± 0.2                 | 4       |             | 2.6 ± 0.6                 | 4       |             |
| Cam5R539T         | 26.4 ± 4.4              | <0.0001 | 51.2        | 3.3 ± 1.4                 | 4       | <0.05       | 26.7 ± 5.2                | 4       | <0.001      |
| CamWT             | 0.4 ± 0.03              | 3       |             | 0.5 ± 0.1                 | 3       |             | 0.9 ± 0.3                 | 3       |             |
| CamWTc580Y        | 8.3 ± 1.1               | <0.0001 | 23.6        | 1.0 ± 0.3                 | 3       | ns          | 11.6 ± 1.4                | 3       | <0.05       |

*aRSA0–3h, ring-stage survival assay from 0 to 3 h; SEM, standard error of the mean; n, number of independent experiments.

bP values were calculated compared to the respective reference line Cam3.IIIrev, V1/Srev, Cam5rev, or CamWT. ns, not significant (P > 0.05).

cFold increase of RSA0–3h percent survival values in mutant-K13 lines compared to their respective isogenic wild-type-K13 lines.

Ozonides. Evidence suggests that ARTs are activated by reductive scission of the endoperoxide bond, catalyzed by free or bound iron in the parasite (35, 43). Potentially, ozonide activation might occur via the same mechanism but might be less efficient, resulting in slower parasite killing. This hypothesis is supported by a study that established a correlation between drug exposure time and activity in vitro. Results showed that shorter (3-h) pulses of DHA, OZ439, and OZ277 yielded a higher percent-survival advantage of lines expressing mutant K13 over lines expressing wild-type K13, compared to their isogenic counterparts expressing the wild-type K13 allele, we observed increased survival of mutant lines at DHA concentrations as low as 2.7 nM (Fig. 2D). The survival advantage of lines expressing mutant K13 over lines expressing wild-type K13 was greater at elevated drug concentrations, where complete inhibition was never observed in the presence of mutant K13. At 700 nM DHA, the IR539T mutation conferred the highest degree of resistance (26.4% in Cam5I543T), followed by R539T (21.5% and 22.7% in Cam3.IIR539T and V1/SR539T, respectively) and finally C580Y (9.2%, 6.4%, and 8.3% in Cam3.IIC580Y, V1/C580Y, and CamWTcC580Y, respectively). This rank order of survival agrees with published data (15).

Interestingly, OZ439 and OZ277 differed substantially in their potency against mutant-K13 ring-stage parasites in the RSA0–3h. OZ439 was fully effective against parasites expressing K13 C580Y or R539T in three different isolates: Cam3.II, V1/S, and
Our results showed reduced potency of OZ439 solely against the Cambodian parasite isolate Cam5 with the K13 I543T mutation (Cam5I543T), which is known to confer high levels of in vitro DHA resistance (compare Fig. 2C to G; Table 1). In contrast with OZ439, the activity of OZ277 was significantly...
impaired by all three K13 mutations analyzed in our study. We found a 2.5- to 13.4-fold increase in parasite survival depending on the K13 mutation and the parasite line (Fig. 2I to L and Table 1). At the highest pulse concentration of 700 nM, we observed an average 21.2% survival when we combined results from six parasite lines harboring one of the three mutant K13 alleles, compared to 3.6% survival in four parasite lines carrying a wild-type K13 allele (Fig. S4). In light of the fact that OZ277 was less potent than DHA against wild-type-K13 parasites and that a substantial number of wild-type-K13 parasites survived drug treatment, the gain of resistance in the presence of K13 mutations was also less pronounced and became significant only at higher concentrations (>87.5 nM).

Two recent studies also reported that OZ277 was compromised by the presence of K13 mutations (39, 44). In both studies, the RSA0–3h 50% was used to assess the susceptibility of mutant-K13 lines to ozonides and to identify potential cross-resistance with DHA. While we found this metric suitable to compare the sensitivity of wild-type-K13 parasites to different drugs, we found it less informative to evaluate mutant-K13 parasites whose dose-response curves differed in a nonconventional way between mutant-K13 and wild-type-K13 lines for all three drugs. At the lower drug concentrations, the dose-response curves overlapped between mutant-K13 and wild-type-K13 lines, whereas at the higher drug concentrations, the sensitive lines showed minimal survival, yet resistant lines reached a plateau of elevated survival that was dose independent. As an example, Cam5Irev and Cam5I543T lines showed comparable profiles at lower concentrations, with RSA0–3h 50% values of 42.2 nM and 41.1 nM, respectively, in contrast with significantly different mean survival values of 2.6% and 26.7%, respectively, at the highest concentration of 700 nM (Fig. 2K). These altered curves were most noticeable when parasites were exposed to DHA, which presumably reflects the underlying mechanism of resistance and kinetics of the pathways involved. To demonstrate how unrepresentative the RSA0–3h 50% values were for DHA resistance in vitro, we compared the RSA0–3h 50% survival value to the RSA0–3h survival value at 700 nM in the Cam3Irev and Cam3I539T lines. The drug concentrations at which 50% inhibition of parasite survival was achieved were 5.8 nM and 4.6 nM, respectively, therefore revealing no major difference, whereas in contrast we observed 0.9% and 21.5% survival, respectively, in these lines exposed to 700 nM DHA. We conclude that the RSA0–3h 50% only partially reflects the ring-stage resistance phenotype (Fig. 2). Our finding that the R539T mutation in Cam3I parasites does not mediate cross-resistance to OZ439 agrees with two other reports (44, 45), whereas a third observed cross-resistance (39). Of note, our R539T data were consistent in both genetic backgrounds tested (Cam3I and V1/S), and our study extends these earlier reports by assessing the roles of K13 mutations across four distinct genetic backgrounds and three mutant-K13 isoforms compared to isogenic wild-type-K13 lines.

Our finding that K13 mutations mediate significant cross-resistance to the first-generation endoperoxide OZ277 is of major concern (Fig. 2I, J, K, and L). Another concern with this short plasma half-life drug is its lack of accumulation in malaria patients even over the course of a recommended 7-day treatment of 150 mg daily (31). In those studies, the mean/peak plasma concentration reached only ~120 nM, which is significantly below the maximal concentration used in our assay and which we observed to be insufficient to inhibit the survival of parasites with wild-type or mutant K13. Its recent registration in India as a licensed drug, in combination with piperaquine, might therefore result in selection for K13 mutants that could compromise both OZ277 and ART efficacy. Indeed, a very recent study from India has detected K13 mutations (notably F446I associated with resistance in Myanmar) (46). OZ277 resistance would in turn place selective pressure on piperaquine, to which resistance is currently spreading in Cambodia on K13 mutant genetic backgrounds, resulting in high levels of treatment failure (47, 48). Monitoring the K13 status and clearance half-lives of Indian infections treated with the combination of OZ277 and piperaquine will therefore be important.

Our findings also provide insight into the suitability of OZ439 as an alternative treatment option, including in Southeast Asia where ACT treatment failures are increas-
ing (1). Studies suggest that its longer half-life (compared to DHA) should suffice to eliminate mutant-K13 parasites in patients, based on cumulative effective doses modeled in Cambodian lines expressing wild-type or R539T K13 (39). Our data provide evidence of nearly equivalent efficacies of OZ439 against mutant-K13 and wild-type-K13 isogenic lines developed on four separate genetic backgrounds, with the exception of the I543T mutation that conferred a degree of protection in the survival assay (although full clearance was nonetheless achieved at high concentrations; Fig. 2G). These data support the ongoing development of OZ439, combined with close scrutiny to test for any selection favoring mutant-K13 parasites, including ones harboring I543T.

**K13 mutations differ in their impact on parasite growth rates in vitro.** Earlier studies with the primary chloroquine resistance determinant PfCRT (*P. falciparum* chloroquine resistance transporter) have shown that fitness costs associated with mutant alleles substantially impacted the prevalence and dissemination of chloroquine-resistant strains and favored alleles with the least fitness defect. Whereas extensive chloroquine use led to a selective sweep of mutant *pfcrt* worldwide, its subsequent removal resulted in less-fit mutant parasites being overtaken by ones expressing wild-type *pfcrt* (42, 49–51). Fitness costs were also observed in an ART drug-pressured mutant *P. falciparum* line, although the molecular basis of resistance was unrelated to K13 and has not been defined (52). We hypothesized that in addition to the broad spectrum of resistance levels conferred by the different K13 alleles, fitness costs conveyed by these mutations may also play an essential role in establishing the viability and distribution of a given haplotype in settings where malaria is endemic. To investigate parasite fitness, we compared in vitro growth rates in competition experiments using isogenic parasite pairs differing solely in their K13 allele status (wild type versus mutant). Since C580Y is the most prevalent allele in Southeast Asia, we selected three different parasite lines carrying this mutation, engineered into two recent Cambodian isolates (Cam3.II and CamWT) as well as the older V1/S line that was culture adapted (in 1976) well before ART was widely deployed clinically. Furthermore, we chose the Cam3.II and V1/S lines that expressed the R539T mutation and extended the study to the I543T mutation expressed in Cam5 parasites.

Growth competition assays were initiated as mixed cultures of isogenic wild-type and mutant parasites in a 1:1 ratio. Genomic DNA was collected every 3 to 4 days over a period of 60 days, and allele frequency was determined via pyrosequencing. This allowed us to calculate the percent changes per 48-h generation of the mutant allele compared to the wild-type allele (Fig. 3). Results showed variable growth defects exerted by K13 mutations on mutant parasites compared to their wild-type-K13 counterparts, with results evoking an important contribution of the parasite genetic background.

These fitness costs were most pronounced in parasites expressing K13 R539T or I543T in comparison with their isogenic wild-type-K13 lines (−0.6% in Cam3.II[R539T], −0.5% in V1/S[R539T], and −1.0% in Cam5[I543T]). These two mutations have previously been observed to confer high levels of *in vitro* drug resistance and significantly prolonged clearance times in patients (10, 13, 15). In contrast, the fitness deficit conveyed by the C580Y allele was essentially nil in two recently culture-adapted Cambodian isolates (−0.06% in Cam3.II[C580Y] and +0.1% in CamWT[C580Y]). The earlier demonstration that C580Y exhibits lower *in vitro* resistance (15) highlights the fact that RSA0−3hsurvival rates alone are not sufficient to predict the spread of individual K13 mutations and evoke an important role for fitness. Our findings are also consistent with the expansion and fixation of this mutation in some parts of Southeast Asia (12, 22, 53). Our evidence that C580Y is the fittest K13 allele is further corroborated by a direct growth competition assay of C580Y with R539T in the Cam3.II background, in which we observed a greater fitness deficit (−0.4%) in parasites expressing K13 R539T compared to an isogenic K13 C580Y line.

In contrast to its lack of a fitness cost in the Cam3.II and CamWT backgrounds, the C580Y mutation in the V1/S strain showed a marked growth defect *in vitro* (−1.1% per
48-h generation) compared to its isogenic wild-type-K13 counterpart (Fig. 3). This suggests an important contribution of the parasite genetic background, which would render the recent Cambodian isolates more receptive to the evolution of ART resistance than older reference lines. These findings support recent population genetics-based studies, which found that resistance to ART evolved de novo on multiple occasions within several ‘founder’ populations present in Cambodia and Vietnam (13, 18, 54, 55).

Interestingly, ART-resistant founder populations have been observed to also contain a suite of additional single nucleotide polymorphisms (SNPs) that are in linkage disequilibrium with K13 (14, 56), suggesting a role in either facilitating or augmenting resistance or enhancing fitness or both (23, 57). Further studies will be required to address other aspects of parasite fitness beyond asexual blood-stage growth rates, notably whether K13 mutations impact gametocytogenesis and transmission to the Anopheles vector. Our data argue that C580Y in particular poses a minimal fitness deficit in its impact on blood-stage proliferation and reinforce the need to monitor the appearance of K13 mutations worldwide and assess their impact on ART efficacy.

MATERIALS AND METHODS

Parasite lines. The parasite lines used herein were described previously (15), with the exception of V1/SC580Y that was developed for this study. In brief, zinc-finger nucleases (ZFNs) directed against K13 (PlasmoDB identifier [ID] PF3D7_1343700, also known as Kelch13) were designed and leveraged to introduce either the wild-type K13 allele or one of three mutant alleles: C580Y, R539T, or I543T. Gene editing was identified by PCR, and clones were obtained by limiting dilution and confirmed by PCR and sequence analysis, as previously described (15).

Parasite cultures. Asexual blood-stage parasites were propagated in human erythrocytes in RPMI 1640 malaria culture medium containing 2 mM L-glutamine, 50 mg/liter hypoxanthine, 25 mM HEPES, 0.225% NaHCO₃, 10 mg/liter gentamicin, and 0.5% (wt/vol) Albumax II (Invitrogen). Parasites were maintained at 37°C under an atmosphere of 5% O₂, 5% CO₂, and 90% N₂.

Ring-stage survival assays (RSA0–3h). The ring-stage survival assays (ring-stage survival assay from 0 to 3 h [RSA0–3h]) were carried out as previously described (15, 40), with minor modifications. In brief, parasite cultures were synchronized 1 or 2 times using 5% sorbitol (Sigma-Aldrich). Synchronous schizonts were incubated for 30 min at 37°C in RPMI 1640 medium supplemented with 25 mM HEPES, 0.225% NaHCO₃, 10 mg/liter gentamicin, and 0.5% (wt/vol) Albumax II (Invitrogen). Parasites were maintained at 37°C under an atmosphere of 5% O₂, 5% CO₂, and 90% N₂.
the drug, as recommended (39). Therefore, we employed a stringent washing procedure involving four consecutive washes with 200 μl of incomplete medium and two transfers of cells to new 96-well plates to ensure complete removal of the drug caused by insufficient washing following the 4-h incubation could result in overestimating drug activity (39). Parasites were then returned to standard culture conditions for an additional 66 h. Parasite survival was assessed by staining with 2× SYBR green I and 165 nM MitoTracker deep red (Invitrogen). Parasitemia was determined by flow cytometry on an Accuri C6 cytometer (58). Between 60,000 and 100,000 cells were examined for each data point. After 72 h, parasitemias had generally expanded to 3 to 5% in DMSO-treated controls, which were used as the reference to calculate the percent survival of drug-treated parasites (40).

**In vitro IC50 assays.** In vitro 50% inhibitory concentrations (IC50s) were determined by incubating parasites for 72 h across a range of concentrations of DHA (0.048 to 100 nM), OZ439 (0.028 to 207.6 nM), or OZ277 (0.39 to 800 nM). Proliferation was determined by flow cytometry (see above). In vitro IC50s were calculated by nonlinear regression analysis, and Student t tests were used for statistical analysis.

**In vitro mixed-culture competition assays, pyrosequencing, and determination of relative growth rates.** For growth competition assays, two parasite lines were mixed 1:1 and seeded in duplicate at an initial parasitemia of 3% ring-stage parasites in drug-free medium. Parasitemia was maintained between 0.3% and 8% to ensure optimal growth conditions. Two to four separate competition assays were performed in duplicate for each parasite pair, and each assay was monitored for an average of 60 days. To determine the ratio of both strains in the mixture over time, saponin-lysed parasite pellets of the mixed cultures were collected on average every 3 or 4 days, and DNA was extracted using DNeasy blood and tissue kits (Qiagen). DNAs were used to determine individual allele frequencies in these mixed cultures by pyrosequencing codon 539, 543, or 580 (see Table S3 in the supplemental material). To calculate the relative growth rates of individual parasite lines, the relative proportion of the two distinct K13 alleles (whose values were always between 0 and 1) were natural log transformed, and linear regression was applied to estimate the relative growth rate value.

**Parasite availability.** Parental and transgenic parasite lines are available through BEI Resources (www.beiresources.org) or upon request from D. A. Fidock.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.00172-17.

**TEXT S1,** DOCX file, 1.2 MB.
**FIG S1,** PDF file, 0.1 MB.
**FIG S2,** PDF file, 0.1 MB.
**FIG S3,** PDF file, 0.1 MB.
**FIG S4,** PDF file, 0.1 MB.
**TABLE S1,** PDF file, 0.1 MB.
**TABLE S2,** PDF file, 0.05 MB.
**TABLE S3,** PDF file, 0.04 MB.

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J.S., N.F.G., and D.A.F. designed the study. J.S., N.F.G., B.H.S., M.E., and A.A.C. generated the experimental data. J.S., N.F.G., and D.A.F. wrote the paper, which was approved by all authors.

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