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Running title: Piperaquine and African *P. falciparum*

We report that *Plasmodium falciparum* resistance to piperaquine, an artemisinin-based combination therapy drug, can be achieved in an African strain via a single PfCRT point mutation. Resistance is modest compared to Asian strains and comes with a substantial fitness cost.
ABSTRACT

Background. Additional therapeutic strategies could benefit efforts to reverse the recent increase in malaria cases in sub-Saharan Africa, affecting mostly young children. A primary candidate is dihydroartemisinin + piperaquine (DHA+PPQ), which is effective for uncomplicated malaria treatment, seasonal malaria chemoprevention, and intermittent preventive treatment. In Southeast Asia, *Plasmodium falciparum* parasites acquired PPQ resistance, mediated primarily by mutations in the *P. falciparum* chloroquine resistance transporter PfCRT. The recent emergence in Africa of DHA-resistant parasites creates an imperative to assess whether PPQ resistance could emerge in African parasites with distinct PfCRT isoforms.

Methods. We edited two PfCRT mutations known to mediate high-grade PPQ resistance in Southeast Asia into GB4 parasites from Gabon. Gene-edited clones were profiled in antimalarial concentration-response and fitness assays.

Results. The PfCRT F145I mutation mediated moderate PPQ resistance in GB4 parasites but with a substantial fitness cost. No resistance was observed with the PfCRT G353V mutant. Both edited clones became significantly more susceptible to amodiaquine, chloroquine, and quinine.

Conclusions. A single PfCRT mutation can mediate PPQ resistance in GB4 parasites, but with a growth defect that may preclude its spread without further genetic adaptations. Our findings support regional use of drug combinations that exert opposing selective pressures on PfCRT.

Keywords: malaria, *Plasmodium falciparum*, antimalarial drug resistance, artemisinin-based combination therapy, piperaquine resistance, PfCRT mutations, genetic editing
INTRODUCTION

Antimalarial drug resistance presents a major barrier to the elimination of Plasmodium falciparum parasites, the causative agent of the most lethal form of human malaria [1]. Malaria control efforts have been further exacerbated by the COVID-19 pandemic, and morbidity and mortality in 2020 rose worldwide to an estimated 241 million cases and 670,000 deaths, predominantly affecting young African children [2].

Artemisinin-based combination therapies (ACTs) became the first-line treatment for P. falciparum in Africa in the 2000s, decades after resistance to the former first-line drugs chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) had swept through the region [3]. ACTs consist of a fast-acting artemisinin derivative that rapidly kills drug-sensitive asexual blood stage parasites yet has a very short plasma half-life (typically <1-2 hr), and a longer-acting partner drug that clears the residual infection [4]. In Africa, the most frequently used ACTs are artemether + lumefantrine and artesunate + amodiaquine. A third ACT, dihydroartemisinin + piperaquine (DHA+PPQ), has shown 99% efficacy in curing uncomplicated malaria in African settings, as per a recent meta-analysis [5, 6]. DHA+PPQ is also proving to be effective for seasonal malaria chemoprevention (defined by the World Health Organization as the intermittent administration of full treatment courses of an antimalarial medicine to children during the malaria season in areas of highly seasonal transmission) as well as intermittent preventative treatment of pregnant women and infants (involving the administration of full therapeutic courses of an antimalarial medicine to pregnant women and infants to prevent adverse consequences of malaria infection), with DHA-PPQ having an advantage over
combinations containing SP that can encounter SP-resistant parasites. DHA-PPQ offers long preventive activity, allowing excellent efficacy when used monthly for chemoprevention. PPQ has the pharmacokinetic advantage of an extended terminal half-life (~20-30 days, compared to ~3 days for lumefantrine), thereby providing post-treatment prophylactic activity [7]. The recent emergence and spread of PPQ resistance in Southeast (SE) Asia [8, 9], however, raises questions about whether PPQ might succumb to resistance in African parasites.

High-grade PPQ resistance can be mediated by mutations in the *P. falciparum* CQ resistance transporter PfCRT, which spans the digestive vacuole membrane [10]. In SE Asia, these mutations arose in strains harboring multicopy *plasmepsins 2 and 3 (pm 2/3)*, which is thought to augment PPQ resistance, as well as mutations in K13 that drive resistance to artemisinin derivatives [11-14]. The spread of PPQ- and DHA-resistant parasites has led to DHA-PPQ treatment failure rates exceeding 50% across several sites in SE Asia [9]. An association between mutant PfCRT and PPQ resistance in vitro, and an increased risk of parasite recurrence post-DHA+PPQ treatment, was first described in Cambodia with the PfCRT F145I mutation [15]. The resistance threshold has been defined in vitro as >10% of ring-stage parasites surviving a 48 hr exposure to 200 nM PPQ, using the PPQ survival assay (PSA) [11, 12]. In vitro resistance is also evidenced by incomplete parasite killing at elevated PPQ concentrations, causing a substantial increase in the IC$_{90}$ (the drug concentration that inhibits parasite growth by 90%) [14, 16]. In Cambodian parasites, this F145I mutation arose in the CQ-resistant PfCRT Dd2 isoform. The Dd2 isoform already carried eight
point mutations relative to the wild-type (3D7) allele (Table 1). F145I, or another
prevalent mutation G353V, conferred PPQ resistance in vitro when edited into the \textit{pfcrt}
locus in Dd2 parasites (a strain that carries a single copy of \textit{pm 2/3}) [16]. Both of these
\textit{PfCRT} mutations substantially reduced parasite growth rates (i.e., caused a fitness
cost).

Here, we have examined whether PPQ resistance might arise in African parasites via
the F145I or G353V mutations in PfCRT. This question is of particular importance given
the recent detection in eastern Africa of parasites that have locally acquired K13
mutations that can cause decreased artemisinin susceptibility [17-20], which in turn
could place additional selective pressure on PPQ as a partner drug [21]. This study
employed a laboratory-adapted African strain, GB4, whose PfCRT isoform is closely
related to the SE Asian Dd2 isoform (the former has only 6 mutations and represents
the most common mutant PfCRT sequence in Africa; Table 1 [22]). Our findings identify
F145I as a causal determinant of moderate PPQ resistance, yet with a fitness cost that
we suspect would hinder its rapid spread in high-transmission African settings. Our
cross-resistance data also suggest a path to mitigate potential resistance through local
use of artesunate-amodiaquine, whose potency is enhanced against our PPQ-resistant
parasites, to treat uncomplicated falciparum malaria.

\textbf{METHODS}

\textbf{Plasmid construction.} Mutations in \textit{pfcrt} (PF3D7_0709000) were edited using
customized zinc-finger nucleases, as previously described [23]. The GB4 donor
template was constructed by site-directed mutagenesis of the plasmid \( \text{pfcrt}^{\text{Dd2-hdhfr}} \) to remove the Dd2-specific N326S and I356T mutations (Supplementary Figure 1A). Additional mutagenesis reactions used primers p1 to p4 to introduce the F145I or G353V mutations (Supplementary Table 1), yielding the plasmids \( \text{pfcrt}^{\text{GB4+F145I-hdhfr}} \) or \( \text{pfcrt}^{\text{GB4+G353V-hdhfr}} \).

Parasite culturing and transfections. GB4 asexual blood stage parasites were cultured in human red blood cells and transfected as described in [23, 24] and detailed in the Supplementary Methods.

In vitro \( \text{IC}_{50} \) drug susceptibility assays. Drug IC\(_{50}\) values were determined for the \( \text{pfcrt} \)-edited lines relative to control lines GB4 (parent strain), Dd2\(^{3D7}\), and Dd2\(^{Dd2+F145I}\). Concentration-response curves were determined for PPQ, CQ, monodesethyl (md)-CQ, md-amodiaquine (md-ADQ), quinine, mefloquine, lumefantrine, DHA, pyronaridine, and ferroquine. Parasites were incubated at 37°C, with 0.2\% starting parasitemias for the lines GB4, GB4\(^{GB4}\), GB4\(^{GB4+G353V}\) and Dd2\(^{3D7}\), and 0.4\% starting parasitemias for GB4\(^{GB4+F145I}\) and Dd2\(^{Dd2+F145I}\), at 1\% hematocrit, across a range of drug concentrations diluted two-fold in 96-well plates. Parasite growth in each well was assessed after 72 h using flow cytometry. Briefly, parasites were stained at 37°C for 20 min with 100 nM MitoTracker Deep Red and 1×SYBR green and quantified on an Accuri C6 flow cytometer [25].
For all antimalarials, in vitro IC₅₀ values were determined by nonlinear regression analysis of concentration-response data with GraphPad Prism 8 software.

**Piperaquine survival assay.** Survival ratios were determined using a modified version of the PPQ survival assay (PSA) [11]. Briefly, 0–6 hr post-invasion (hpi) ring-stage parasites, which had been doubly synchronized with sorbitol (5% final volume, performed ~18 hr apart in the same cycle), were incubated at 1.0% starting parasitemia and 1% hematocrit with varying concentrations of PPQ (tetraphosphate tetrahydrate salt; Alfa Aesar) at 37°C for 72 hr in 96-well plates. PPQ was dissolved in 0.5% lactic acid in water. The assay was carried out over the range 1.6 nM – 1,600 nM PPQ. Parasitemias were determined via flow cytometry. Assays were repeated independently six times in duplicate or triplicate (Figure 1A; Supplementary Table 2). Statistical comparisons employed Mann–Whitney U tests. PPQ survival was defined as the percentage of parasites remaining relative to the no-drug control (100% survival).

**Competitive fitness and TaqMan allelic discrimination assays.** Fitness assays were performed by co-culturing synchronized trophozoites of isogenic wild-type (GB4) and mutant edited parasites in 1:1 ratios. Cultures were maintained in 6-well plates and sampled every four days over a period of 32 days. Parasite samples were freed from their red blood cells using 0.1% saponin and genomic DNA was extracted using the QIAmp DNA Blood Mini Kit. The percentage of the wild-type or mutant allele in each sample was determined using TaqMan allelic discrimination real-time PCR assays as described in [25, 26] and detailed in the Supplementary Methods.
Structural modeling. The single-particle cryo-EM structure of the PfCRT 7G8 isoform (PDB:6UKJ; [27]) was imported into the Schrödinger molecular modeling suite (Maestro, version 2019-1). The PfCRT protein structure was analyzed using the Protein Preparation Wizard. Force field atom types and bond orders were assigned, missing atoms including hydrogens were added, tautomer/ionization states were assigned, the hydrogen bond network was optimized, and a constrained energy minimization was performed. F145I and G353V were introduced into the PfCRT 7G8 structure using the Residue and Loop Mutation tool, and then refined by a local minimization step. The final structure was visualized in Schrödinger PyMOL to display the residues of interest in specific colors.

PfCRT haplotype analysis. This publication uses data from the MalariaGEN Plasmodium falciparum Community Project as described in [28].

RESULTS

PfCRT F145I mediates PPQ resistance in GB4 (Gabon) parasites

To evaluate the effect of the PPQ resistance-conferring PfCRT mutations (F145I or G353V) on an African P. falciparum strain, we used zinc-finger nucleases to genetically edit these mutations into the pfcr1 locus in the CQ-resistant GB4 line, yielding GB4^{GB4+F145I} and GB4^{GB4+G353V} (Table 1; Supplementary Figure 1). We also generated a control line (GB4^{GB4}) edited to express the parental isoform.
To test for PPQ resistance, we employed the PSA whereby survival rates are calculated after exposing 0-6 hr ring-stage parasites to PPQ [11]. As a control, we tested in parallel the pfcrt-edited PPQ-resistant Dd2\textsubscript{Dd2+F145I} line, which is an Asian parasite expressing the Dd2+F145I isoform. For GB4\textsubscript{GB4+F145I}, 8% survival was observed at 200 nM PPQ, and >10% survival was identified at higher concentrations (400 to 1600 nM). These survival rates were lower than Dd2\textsubscript{Dd2+F145I} (21-24% survival at 200 to 1600 nM PPQ) but were significantly higher than GB4 or GB4\textsuperscript{GB4} (showing a background of 1% survival at these high concentrations). In contrast, GB4\textsubscript{GB4+G353V} showed no increased survival at high PPQ concentrations, with a significant increase only evident at 50 nM or lower (Figure 1A; Supplementary Table 2).

Morphologically, we also observed enlarged, translucent digestive vacuoles lined by hemozoin crystals in both the trophozoite and schizont stages of GB4\textsubscript{GB4+F145I} parasites expressing the GB4+F145I isoform. A similar phenotype was observed with PPQ-resistant Dd2\textsubscript{Dd2+F145I} parasites (Figure 1B). Edited GB4\textsubscript{GB4+G353V} showed only slightly bloated digestive vacuoles compared with GB4 and GB4\textsuperscript{GB4}, in contrast to the distended digestive vacuoles previously observed in the PPQ-resistant Dd2\textsubscript{Dd2+G353V} line [16].

We also measured PPQ susceptibility using 72 hr concentration-response assays with asynchronous parasites. Results showed sustained parasite growth at elevated PPQ concentrations in the GB4\textsubscript{GB4+F145I} line, although this was less pronounced than with Dd2\textsubscript{Dd2+F145I}. No such phenotype was observed with GB4\textsubscript{GB4+G353V}, confirming its lack of resistance (Supplementary Figure 2; Supplementary Table 2). It is important to note...
the essentiality of the PSA for determining and quantifying PPQ resistance, since both GB4\textsuperscript{GB4+F145I} and GB4\textsuperscript{GB4+G353V} had lower PPQ IC\textsubscript{50} values in this standard concentration-response assay when compared with the isogenic control line GB4\textsuperscript{GB4}.

These data are consistent with earlier observations that PPQ resistance is only apparent at high drug concentrations, a phenomenon that we suspect is related to concentration-dependent engagement of drug efflux via mutant PfCRT [16, 23, 29].

Piperaquine-resistant GB4 parasites manifest hypersensitivity to 4-aminoquinoline drugs

Edited pfcr\textsuperscript{t} variants were next profiled against a panel of licensed antimalarial drugs in 72 hr concentration-dependent growth inhibition assays (Supplementary Table 3). Results showed that when compared with the isogenic control line GB4\textsuperscript{GB4}, the mutant GB4\textsuperscript{GB4+F145I} line showed increased susceptibility to md-ADQ, the active metabolite of this African ACT partner drug, manifesting as a 2-fold lower mean IC\textsubscript{50} value (Figure 2A). A similar increase in md-ADQ sensitivity was observed in the GB4\textsuperscript{GB4+G353V} line, despite this line not acquiring PPQ resistance. Both PfCRT variants also resulted in GB4 parasites becoming significantly more sensitive to quinine, used as a treatment for severe malaria (Figure 2B). We also observed that both mutations reversed GB4 parasite resistance to CQ and its metabolite md-CQ. This sensitization was most pronounced with the GB4\textsuperscript{GB4+F145I} line, which became as sensitive to CQ and md-CQ compounds as the comparator Dd2\textsuperscript{3D7}, a Dd2 line expressing the 3D7 wild-type PfCRT isoform (Figure 2C, 2D). There was no significant difference in mean IC\textsubscript{50} values for GB4\textsuperscript{GB4+F145I} and GB4\textsuperscript{GB4+G353V}, compared to the GB4 controls, with the other ACT drugs
mefloquine (MFQ), lumefantrine (LMF), DHA, or pyronaridine (PND). Similarly, there was no IC\textsubscript{50} shift against ferroquine (FQ), presently in human clinical trials as a novel antimalarial [30] (Supplementary Figure 3A-E).

Piperaquine-resistant GB4+F145I parasites display a substantial growth defect

To determine the relative asexual blood stage fitness conferred by the F145I and G353V mutations on the African GB4 background, we co-cultured edited mutant parasites with the wild-type GB4 editing control in pairwise competitive assays (Figure 3A). The relative expansion of each line was assessed by TaqMan-based allelic discrimination quantitative PCR (qPCR) (Supplementary Tables 1 and 4). Assays were initiated with tightly synchronized trophozoites mixed in a 1:1 ratio of wild-type to mutant isogenic parasites, and cultures were maintained over a period of 32 days (~16 generations of asexual blood stage growth). GB4\textsuperscript{GB4+F145I} parasites were fully outcompeted by GB4\textsuperscript{GB4} within 12 days (~6 generations), illustrating a substantial fitness cost of the F145I mutation on edited GB4 parasites (Figure 3A). From these data, we calculated the fitness cost, which represents the percent reduction in growth rate per 48 hr generation of a test line compared to its wild-type isogenic comparator. For GB4\textsuperscript{GB4+F145I}, the mean fitness cost was measured at 39.2% per generation (Figure 3B). However, the G353V mutation had no effect on the fitness of GB4 parasites (with only a 0.2% fitness cost). These data revealed that the high PSA survival rate of GB4\textsuperscript{GB4+F145I} parasites was offset by a high fitness cost (Figure 3B).
DISCUSSION

PPQ presents considerable advantages as an additional partner drug for antimalarial combination therapy and chemoprevention in Africa due to its potency and long half-life. The emergence of resistance in SE Asia, however, calls for investigations into the risk of resistance arising in genetically-distinct African parasites. Here, we provide evidence that moderate PPQ resistance can be acquired via a single point mutation, F145I, introduced into the widely prevalent mutant GB4 isoform of the CQ resistance transporter, PfCRT. This gain of resistance was accompanied by a very substantial loss of parasite fitness, which we posit could preclude this mutation from being competitive in high-transmission African settings with typically mixed infections. We also find that PPQ resistance results in increased sensitization to ADQ, quinine and CQ, creating a rationale for implementing regimens with different drug combinations that exert opposing selective pressures on parasite populations and thereby hinder the spread of multidrug-resistant *P. falciparum* malaria [23].

Recent reports of mutant *k13* alleles in local parasite populations in Rwanda and Uganda provided a major impetus for our study. These include clinical evidence of delayed parasite clearance following treatment with an ACT and in vitro evidence of ART resistance [17-20]. Recent data suggest that ART resistance mediated by K13 mutations does not extend to all African strains, implying an important contribution of genetic background [31]. Nonetheless, ART resistance in Africa is likely to spread, thereby imposing greater selective pressure on ACT partner drugs. Lumefantrine has not yet encountered resistance, and ADQ still remains widely effective, with these drugs exerting selective pressure in favor of wild-type and mutant *pfcr* alleles, respectively.
As PPQ is increasingly adopted as a curative and chemopreventive drug in Africa, the lessons learnt from SE Asia can help guide its use in ways that mitigate the risk of resistance.

High-grade PPQ resistance in SE Asia is driven primarily by point mutations in PfCRT, with F145I conferring the greatest degree of resistance. In the Asian Dd2 strain, this mutation was earlier found to confer a substantial fitness cost, estimated at ~17% per 48 hr generation of asexual blood stage growth, and in the field other mutations with reduced fitness costs have gained in prevalence (such as T93S) [29, 33]. These PfCRT mutations arose in SE Asian strains that express multiple copies of pm 2/3. These hemoglobinases are thought to augment PPQ resistance [14] and potentially alleviate some of the mutant PfCRT-mediated fitness costs. Of note, parasites harboring multicopy pm 2/3 have yet to be detected in African studies [19, 34, 35]. In SE Asia, PPQ-resistant parasites also typically harbor mutant K13 [36]. The pathway unifying these resistance determinants is hemoglobin catabolism, used by the parasites to generate peptides for protein synthesis, but also an Achilles heel as it generates toxic heme byproducts to which PPQ, CQ, and ADQ bind to prevent detoxification [37]. This process occurs in the parasite’s acidic digestive vacuole, where these drugs accumulate and where pm 2/3 help proteolytically cleave hemoglobin [10]. PfCRT is known to efflux both globin-derived peptides as well as drugs depending on the structural conformation of the isoform. Impaired peptide flux is a feature common to mutant PfCRT isoforms [38-41]. Morphologically, this manifests as distended translucent digestive vacuoles [16], as seen herein with GB4 and Dd2 parasites expressing the GB4+F145I and
Dd2+F145I mutant PfCRT isoforms, respectively. K13 is also connected as it regulates the import of host hemoglobin into newly-formed ring-stage parasites, and mutations conferring ART resistance act in part to restrict this endocytic process [42, 43].

In African GB4 parasites expressing the GB4+F145I variant, we estimate a fitness cost of ~39% reduced growth rate per generation compared with the isogenic parasites expressing the parental GB4. By comparison, parental GB4 itself also produced a fitness cost of 8% slower growth per generation compared with the wild-type isoform in isogenic parasites [22]. Earlier studies in Malawi, later confirmed across Africa, showed that the fitness costs observed with CQ-resistant PfCRT isoforms was sufficient for wild-type PfCRT parasites to overtake mutant isolates once CQ was no longer used clinically [44]. In The Gambia, studies showed that this attrition of mutant PfCRT could occur within a single season of malaria transmission [45]. The impact of these reduced growth rates on parasite viability at the population level is thought to be exacerbated in high-transmission African settings with frequently mixed infections, robust host immunity, and reduced per-patient use of therapeutics in the adult population, as compared to SE Asian settings [46]. These observations lead us to surmise that the GB4+F145I variant alone would not be competitive in African epidemiological settings.

Strikingly, no change in PPQ susceptibility was observed upon introducing the PfCRT G353V mutation into GB4 parasites, despite its demonstrated ability to mediate PPQ resistance in Asian strains [22]. Of note, the GB4 and Dd2 PfCRT isoforms differ at the nearby position 356, which highlights the potential influence of PfCRT amino acids on...
nearby residues and overall protein function (Supplementary Figure 4). These isoforms also differ at residue 326, which in its wild-type state as found in GB4 increases ADQ susceptibility when compared with the Dd2 N326S mutation [47]. Of note, molecular dynamics simulations based on the recently elucidated PfCRT structure provided evidence that the F145I mutation may change the conformation of helices surrounding the PfCRT binding cavity, thereby influencing drug and peptide efflux properties [27].

Our findings of increased sensitization to ADQ in our PPQ-resistant GB4\textsuperscript{GB4+F145I} line evokes consideration of the potential benefits of combining these two drugs into a triple ACT. A similar approach, combining artemether+lumefantrine with ADQ, recently showed excellent efficacy in Cambodia and is being considered to prolong the therapeutic lifetime of artemether+lumefantrine [48]. An alternative would be to allow multiple first-line therapies to be used concurrently in local settings, such as DHA+PPQ and artesunate-ADQ that would exert opposing selective pressures on PfCRT variants [49].

Our study demonstrates that PPQ resistance can arise on an African background from acquisition of a single point mutation in PfCRT added to the CQ-resistant GB4 mutant isoform, albeit with a severe fitness cost. This GB4 isoform was earlier estimated to have a 19% prevalence in a collection of 783 sequenced African isolates present in the Pf3K data set [22, 50]. The next most common mutant PfCRT isoform, termed Cam783, had a 12% prevalence, with the CQ-sensitive wild-type (3D7) allele present in 66% of
samples. Our analysis of PfCRT haplotypes in the Pf6 data set, comprising 3,885 African samples, shows that mutant PfCRT (as per the available partial haplotype data) remained quite common across Western, Central and Eastern Africa, unlike Southern Africa where almost all parasites expressed wild-type PfCRT (Supplementary Figure 5). Further studies will be important to test additional PfCRT mutations and background haplotypes [22] to fully assess the possibility of PPQ resistance arising in Africa.

POTENTIAL CONFLICTS OF INTEREST
All authors declare no conflict of interest.

SUPPORTING INFORMATION
Partial funding for this work was provided by the National Institute of Allergy and Infectious Diseases at the National Institutes of Health (grant numbers R01 AI050234, R01 AI146728, R01 AI124678, R21 AI127581 to DAF; R01 AI143521 to KJW; F31 AI157410 to LMH; and K08 AI163497 to JLSS). JLS gratefully acknowledges support from a Doris Duke Physician Scientist Award and a Louis V. Gerstner, Jr. Scholars Award. LMH was funded in part by the Columbia University Graduate Training Program in Microbiology and Immunology (T32 AI106711; Program Director D. Fidock).

PRIOR DATA PRESENTATION
A part of Figure 1A was presented during an overview talk by DAF at the Keystone Symposium on Malaria, held in Breckenridge, CO, April 2022.
REFERENCES


FIGURE LEGENDS

Figure 1. GB4+F145I PfCRT confers high-grade PPQ resistance. (A) PPQ survival assay data for parental and pfcrte-dited parasite lines displayed as concentration-response curves. Dd2 and GB4 lines harboring the F145I mutation showed a survival rate of 21.3% and 7.8%, respectively at the reference concentration of 200 nM, with the former exceeding the 10% cutoff earlier proposed to be indicative of in vitro resistance [12]. Assays were conducted on six separate occasions with 2-3 technical replicates. Significance between pfcrte-dited clones and their parental controls (GB4 or Dd2) was determined using Mann–Whitney U tests; **p<0.01; ***p<0.001 for GB4 and Dd2 edited lines compared to their isogenic control lines. (B) Cell morphology of parental and pfcrte-dited parasite lines. Images reveal enlarged, translucent digestive vacuoles to various degrees in parasites expressing novel PfCRT variants with F145I and G353V mutations.

Figure 2. Concentration-response data for antimalarial drugs profiled against parental and pfcrte-dited parasite lines. Results show complete or partial reversal of resistance and increased sensitivity of GB4 parasites harboring the PfCRT mutations F145I and G353V, relative to the control lines and the Dd2 parasite expressing wild-type 3D7 against the drugs (A) monodesethyl-amodiaquine (md-ADQ); (B) quinine (QN); (C) chloroquine (CQ); and (D) the CQ active metabolite monodesethyl-CQ (md-CQ). Comparisons are shown between pfcrte-dited parasites and their isogenic GB4 or Dd2 controls. Significance was determined using Mann–Whitney U tests with N,n = 4,2; *p<0.05.
Figure 3. GB4\(^{GB4+F145I}\) exhibits a significant fitness cost. (A) Parasite fitness was determined by pairwise competition of either mutant PfCRT-edited line, F145I and G353V, with an isogenic wild-type control over 32 days. Percentages of mutant alleles relative to the wild-type allele overtime were determined using custom Taqman allelic discrimination qPCR assays. Results, shown as mean ± SEM, were obtained from three independent experiments, each performed in duplicate. (B) PSA survival rates at 200 nM PPQ (left Y axis, in red) and mean relative fitness costs per generation (right Y axis, in blue) are shown for GB4\(^{GB4+F145I}\) and GB4\(^{GB4+G353V}\) relative to parental GB4\(^{GB4}\).
Table 1. PfCRT haplotypes for parasite lines used in this study

<table>
<thead>
<tr>
<th>Parasite line&lt;sup&gt;a&lt;/sup&gt;</th>
<th>edited</th>
<th>PfCRT Haplotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dd2&lt;sup&gt;3D7&lt;/sup&gt; (WT PfCRT)</td>
<td>Yes</td>
<td>M N K F A Q N G I R</td>
</tr>
<tr>
<td>Dd2 (parent)</td>
<td>No</td>
<td>I E T F S E S G T I</td>
</tr>
<tr>
<td>Dd2&lt;sup&gt;Dd2+F145I&lt;/sup&gt;</td>
<td>Yes</td>
<td>I E T I S E S G T I</td>
</tr>
<tr>
<td>GB4 (parent)</td>
<td>No</td>
<td>I E T F S E N G I I</td>
</tr>
<tr>
<td>GB4&lt;sup&gt;GB4&lt;/sup&gt; (editing control)</td>
<td>Yes</td>
<td>I E T F S E N G I I</td>
</tr>
<tr>
<td>GB4&lt;sup&gt;GB4+F145I&lt;/sup&gt;</td>
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<tr>
<td>GB4&lt;sup&gt;GB4+G353V&lt;/sup&gt;</td>
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<td>I E T F S E N V I I</td>
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<sup>a</sup>Dd2<sup>Dd2</sup> (the pfcr<sub>c</sub> editing control) is not listed, as this study only used Dd2<sup>3D7</sup> and Dd2<sup>Dd2+F145I</sup> for statistical comparisons; WT, wild-type.
Figure 1

A

% parasite survival

100
10
1
0.1

[ Piperaquine ] nM

GB4 parent
GB4 GB4
GB4 GB4 GB4 F145I
GB4 GB4 GB4 G352V
Dd2 3D7
Dd2 Dd2 Dd2 F145I

B

Trophozoites

Schizonts

GB4 parent
GB4 GB4
GB4 GB4 F145I
GB4 GB4 G352V
Dd2 3D7
Dd2 Dd2 F145I

Figure 1

134x163 mm (x DPI)
Figure 2

A

B

C

D

md-ADQ IC₅₀ (nM)

QN IC₅₀ (nM)

CQ IC₅₀ (nM)

md-CQ IC₅₀ (nM)

GB4 parent

GB4 ΔAB1

GB4 Δ365V

D2ΔD7

D2ΔD7;ΔAB1

114x133 mm (x DPI)
Figure 3

A

% mutant parasites

FIT

UNFIT

Day

0 8 16 24 32

B

PSA survival rate (%)

Fitness cost (%) per generation (relative to GB4)

GB4 GB4+G353V

GB4 GB4+F145I

Figure 3

112x174 mm (x DPI)