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Balancing drug resistance and growth rates via compensatory mutations in the *Plasmodium falciparum* chloroquine resistance transporter

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Summary

The widespread use of chloroquine to treat *Plasmodium falciparum* infections has resulted in the selection and dissemination of variant haplotypes of the primary resistance determinant PfCRT.

These haplotypes have encountered drug pressure and within-host competition with wild-type drug-sensitive parasites. To examine these selective forces *in vitro*, we genetically engineered *P. falciparum* to express geographically diverse PfCRT haplotypes. Variant alleles from the

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Author Contributions

Conceived and designed the experiments: IP AML DAF. Performed the experiments: IP AE REL AML MJA JS PHH EP DJJ OCF CS AML. Analyzed the data: IP SJG GLJ AE SKD AML ML DAF. Wrote the paper: IP SJG GLJ AML DAF.

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Philippines (PH1 and PH2, which differ solely by the C72S mutation) both conferred a moderate gain of chloroquine resistance and a reduction in growth rates *in vitro*. Of the two, PH2 showed higher IC₅₀ values, contrasting with reduced growth. Furthermore, a highly mutated *pfcr* allele from Cambodia (Cam734) conferred moderate chloroquine resistance and enhanced growth rates, when tested against wild-type *pfcr* in co-culture competition assays. These three alleles mediated cross-resistance to amodiaquine, an antimalarial drug widely used in Africa. Each allele, along with the globally prevalent Dd2 and 7G8 alleles, rendered parasites more susceptible to lumefantrine, the partner drug used in the leading first-line artemisinin-based combination therapy. These data reveal ongoing region-specific evolution of PfCRT that impacts drug susceptibility and relative fitness in settings of mixed infections, and raise important considerations about optimal agents to treat chloroquine-resistant malaria.

Keywords

malaria; PfCRT; drug accumulation; chloroquine resistance; fitness; transfection

Introduction

In functionally constrained genes, the rise of non-synonymous mutations may decrease an organism's fitness by steering it away from a long-optimized machinery of closely interacting components. Without a specific selective pressure, the more fit wild-type usually predominates at the population level and the less-fit variants are either eliminated or persist at low frequencies (Mitchell-Olds *et al.*, 2007). An example of selective pressure is the use of drugs to treat human pathogens. As such, these constitute an ideal group in which to study the balance between surviving drug pressure and remaining competitive with wild-type organisms.

Generally, fitness costs associated with initial resistance-conferring mutations will lead to the attrition of mutants upon the removal of drug pressure, allowing for the reemergence of surviving wild-type organisms (Hastings and Donnelly, 2005). However, prolonged drug exposure can provide pathogens the opportunity to acquire additional mutations, either within the primary resistance determinant or within secondary factors, which compensate for the initial fitness cost (Brown *et al.*, 2010). Competition between mutants carrying those compensatory mutations can then take place, leading to a mutant population with a reduced fitness cost that eventually can successfully compete with wild-type organisms in a drug-free environment (Levin *et al.*, 2000). Fitness costs and the prevalence of initial resistance-conferring mutations can also be influenced by the use of other drugs to replace the failed therapeutic agent, particularly in instances where their efficacy is impacted by the same resistance determinants.

The malarial parasite *Plasmodium falciparum*, which caused an estimated 198 million clinical cases and 584,000 deaths in 2013 (WHO, 2014), is a prime example of a human pathogen that repeatedly encounters drug pressure and within-host competition among parasite strains. For much of the 20th century, the antimalarial treatment of choice was chloroquine (CQ), a drug characterized by its rapidity of action, safety and low cost. This

drug prevents the detoxification of reactive iron-containing heme that is liberated as a result of hemoglobin proteolysis in the acidic digestive vacuole (DV) of intra-erythrocytic parasites (Fitch, 2004). CQ resistance (CQR) emerged slowly, but by the early 1990s had taken hold across virtually all the malaria-endemic world (Wellems and Plowe, 2001). The cellular basis for CQR has been attributed to reduced CQ accumulation in the DV, resulting in diminished access of CQ to its otherwise toxic heme target (Saliba *et al.*, 1998).

At the molecular level, CQR has been traced primarily to amino acid changes in the DV transmembrane protein PfCRT (Fidock *et al.*, 2000). These include K76T, ubiquitous among CQ-resistant strains and a highly sensitive marker of CQ treatment failure, as well as 3–8 additional PfCRT polymorphisms that produce region-specific haplotypes (Ecker *et al.*, 2012). These haplotypes reflect a handful of origins of mutant *pfcr*t that disseminated under drug pressure in selective sweeps across the world (Nash *et al.*, 2005; Kidgell *et al.*, 2006; Mu *et al.*, 2010; Park *et al.*, 2012). At least 34 different mutant PfCRT haplotypes have been reported, contrasting with a conserved wild-type haplotype in CQ-sensitive parasites (Isozumi *et al.*, 2010; Ecker *et al.*, 2012; Baro *et al.*, 2013). In Malawi, the prevalent African mutant form of PfCRT (CVIET haplotype at positions 72–76, found in strains including Dd2) largely disappeared within several years of CQ withdrawal, presumably due to a fitness cost that rendered this variant less competitive than *pfcr*t wild-type parasites in the absence of drug pressure (Kublin *et al.*, 2003; Mita *et al.*, 2003; Laufer *et al.*, 2010).

The notion that mutation of PfCRT negatively impacts parasite fitness in field settings is supported by recent *in vitro* metabolomic and allelic competition investigations, which revealed a defect in hemoglobin catabolism and reduced relative growth rates *in vitro*, interpreted as a proxy of fitness costs for the CQ-resistant Dd2 and 7G8 PfCRT haplotypes (Lewis *et al.*, 2014). Consistent with these findings, a fitness disadvantage was observed for CQ-resistant parasites during the dry season in The Gambia, when drug pressure is transiently removed and transmission is low (Ord *et al.*, 2007). This fitness disadvantage was recapitulated in southern Zambia at the level of vectorial selection, whereby the wild-type (K76) form of PfCRT was significantly enriched in the infected *Anopheles arabiensis* mosquitoes compared to its baseline prevalence in the local infected human populations (Mharakurwa *et al.*, 2013). Field studies in South America and Asia, however, have documented no or only modest attrition in mutant PfCRT forms, including 7G8 (SVMNT haplotype at positions 72–76), despite the discontinued use of CQ for the treatment of *P. falciparum* malaria for over two decades (Wang *et al.*, 2005; Chen *et al.*, 2008; Griffing *et al.*, 2010). Those studies have led to the suggestion that the fitness cost of parasites harboring the ⁷²SVMNT⁷⁶ haplotype may be less severe than that of parasites carrying the ⁷²CVIET⁷⁶ signature (Sa and Twu, 2010).

Aside from regional differences in the choice of antimalarial drug regimens that may help sustain variant PfCRT haplotypes (Ecker *et al.*, 2012), studies of human and murine parasites highlight a potential selective advantage of mutant *pfcr*t alleles in enhancing human to mosquito transmission of parasites following CQ treatment. Among Sudanese parasite isolates bearing the K76T mutation in PfCRT, a higher gametocyte carriage rate was observed as compared to parasites encoding wild-type PfCRT (Osman *et al.*, 2007). Furthermore, *P. berghei* parasites engineered to express the *P. falciparum* 7G8 *pfcr*t variant

protected early gametocytes against CQ action and were transmitted at higher levels compared to drug-sensitive parasites (Ecker *et al.*, 2011). These observations underscore the complexity of factors that collectively determine the fitness of *Plasmodium* parasites, of which relative growth rates in infected erythrocytes is but one component, with others including antimalarial drug susceptibility profiles, the impact of host immunity and transmission dynamics, differences in gametocyte production and infectivity, competition between strains within mosquitoes, and growth differences that could manifest during the liver stages (Walliker *et al.*, 2005; Rosenthal, 2013).

Here, we dissect the specific contribution of geographically distinct PfCRT haplotypes to parasite fitness and antimalarial drug susceptibility. Our study includes novel haplotypes that have not been previously assessed in a controlled genetic background, including two closely-related PfCRT isoforms from the Philippines (Chen *et al.*, 2005), as well as an allele from Cambodia that harbors nine mutations, an exceptionally high number (Durrand *et al.*, 2004). These alleles were assessed alongside the geographically widespread Dd2 and 7G8 alleles *in vitro* in drug susceptibility assays as well as mixed-infection competition assays. We also investigated how various mutant PfCRT haplotypes impact CQ accumulation and parasite response to other antimalarials in current clinical use. Our results highlight the importance of regional PfCRT haplotypes in contributing to parasite fitness and define a novel allele in Cambodia that appears to have overcome the hurdle of reduced fitness associated with less mutated *pfert* forms, while still maintaining a moderate degree of CQR.

Results

Generation of Isogenic Parasite Lines Expressing Asian *pfert* Alleles From the Endogenous Locus by Allelic Exchange

We engineered the mutant *pfert* alleles PH1 and PH2 (from the Philippines) and Cam734 (from Cambodia) into *P. falciparum* CQ-sensitive parasites via allelic exchange. The recipient CQ-sensitive strain C1^{GC03} was genetically modified from the GC03 parasite line, a progeny of the HB3 × Dd2 genetic cross (Su *et al.*, 1997), in a prior round of transfection. Briefly, the highly interrupted endogenous wild-type *pfert* gene sequence was replaced with a shortened sequence containing all exons and intron 1, rendering this line more amenable to *pfert* allelic exchange (Sidhu *et al.*, 2002). PH1 and PH2 represent two common PfCRT haplotypes in the Philippines (Chen *et al.*, 2003) that differ from one another at position 72 and that are notable for lacking the common A220S mutation but harboring the two novel mutations A144T and L160Y (Table 1). Cam734 comprises ~20% of all *pfert* alleles in Cambodia and is a highly mutated *pfert* allele, differing from the wild-type allele at 9 positions (Durrand *et al.*, 2004).

To generate recombinants, C1^{GC03} parasites were electroporated with the plasmids pBSD-crt-PH1^{Py3'}, pBSD-crt-PH2^{Py3'}, and pBSD-crt-Cam734^{Py3'}, containing exons 2–13 of the three Asian *pfert* alleles (Fig. 1A). Transfected parasite cultures were obtained following exposure to blasticidin and WR99210 to select for expression of *blasticidin S-deaminase* (*bsd*) from the transfection plasmid and human *dihydrofolate reductase* (*dhfr*) in the C1^{GC03} parental line, respectively.

PCR was used to identify transfected lines that had undergone homologous recombination and single-site crossover into the *pfert* locus. Recombinant clones were then obtained by limiting dilution. Clones from each successfully integrated transfection were selected for further characterization and were termed C8^{PH1-I}, C8^{PH1-II}, C10^{PH2-I}, C10^{PH2-II}, C12^{Cam734-I}, and C12^{Cam734-II}. Following our earlier reports (Sidhu *et al.*, 2002), the superscript indicates the *pfert* allele, with the Roman numeral indicating the clone. For comparison, we included C1^{GC03}, which was generated using the same allelic exchange strategy (Sidhu *et al.*, 2002) and which expresses the canonical wild-type allele (Table 1).

To confirm the clonality of these lines, we performed PCR with primers P1 and P3 that targeted parasites with integrated plasmid, and primers P1 and P2 that were specific for the original C1^{GC03} locus (Fig. 1A, 1B; Table S1). Primers P1 and P3 yielded the expected 1.3 kb band from the clones that had undergone two rounds of recombination, but not from the first-round C1^{GC03} or parental GC03 parasites. PCR with primers P1 and P2 resulted in expected bands of 3.3 kb from the unmodified genomic *pfert* locus in GC03 and 1.7 kb from the first round of recombination present in the C1^{GC03} line (Fig. 1B). Southern blot analysis of genomic DNA (gDNA) digested with SalI+ClaI revealed band sizes of ~16.3 kb, 8.1 kb, 7.7 kb and 1.2 kb, consistent with plasmid integration into the C1^{GC03} *pfert* locus (Fig. 1C). GC03 parasites and linearized plasmid DNA showed the predicted 9.4 kb and 7.7 kb bands, respectively.

Sequencing of the functional recombinant *pfert* locus amplified from gDNA, which was performed shortly after limiting dilution cloning, confirmed the expected full-length sequence of *pfert* in the individual lines. Amplification of the *pfert* locus from cDNA and gDNA and subsequent sequencing of the polymorphic region encoding for amino acids 72–76 confirmed the exclusive expression of the integrated allele in the new second-round recombinants (data not shown). Real-time PCR analysis was also performed using two independent preparations of parasite RNA from synchronized ring-stage cultures. These were assayed for *pfert* and the housekeeping gene *actin* (PFL2215w) on 4–8 independent occasions with each sample tested in triplicate per assay. Kruskal-Wallis tests showed no significant differences in *pfert* transcript levels between any *pfert*-modified lines (Table S2). The same finding of statistically indistinguishable expression levels was observed by quantitative Western blot analysis of protein extracts from these recombinant lines (Table S2).

Ongoing characterization of these recombinant lines, during the lengthy period of propagation required to complete their phenotypic assessment, revealed a highly unusual event in the C8^{PH1-I} clone. Over time, a subpopulation arose that outgrew the original line. This subpopulation was found to have undergone reversion of the N326D mutation back to the wild-type N326 codon in *pfert*, with the other three PH1 mutations being retained (K76T, A144T and L160Y; Table 1). Interestingly, this revertant was found to be CQ sensitive, implicating N326D as an important contributor to CQR, consistent with a previous report (Summers *et al.*, 2014). Nevertheless, the advent of a spontaneous sequence reversion in this line made us cautious about using the C8^{PH1-I} line, and it was excluded from further consideration in this present study. Repeated sequence analysis of other recombinant lines during long-term culture confirmed the genotypes of all other lines under investigation.

Analysis of the PH1 haplotype was therefore confined to the C8^{PH1-II} clone, whose genotype was closely monitored and remained stable over time.

Mutant Southeast Asian *pfcr*t Alleles Influence Susceptibility To Locally Used Drugs

Using these recombinant isogenic lines, we assayed the impact of different PfCRT haplotypes (Table 1) on parasite susceptibility to CQ as well as other antimalarials. For comparison, we included C2^{GC03}, C4^{Dd2}, and C6^{7G8}, which were generated using the same genetic strategy (Sidhu *et al.*, 2002). These encode the wild-type GC03 haplotype, the Dd2 haplotype commonly found in Asia and Africa, and the 7G8 haplotype that is widespread in South America and the Pacific region, respectively (Sa *et al.*, 2009). For reference, we also included the non-recombinant lines GC03, Dd2 and 7G8.

We note that our CQ values, both for resistant and sensitive strains, are lower than earlier reports (Sidhu *et al.*, 2002; Lakshmanan *et al.*, 2005; Valderramos *et al.*, 2010). One important technical difference is that we reduced the HEPES concentration from the earlier 50 mM to the current 25 mM. Our detailed studies have since revealed that this decrease in the HEPES concentration leads to a substantial reduction in half-maximal inhibitory concentration (IC₅₀) values for CQ-sensitive and even more so for CQ-resistant parasites, as detailed in the Supporting Information (SI; see text and Figures S2–S4). This is one of the variables that can produce differences in CQ IC₅₀ values (others include genetic differences between strains maintained long-term in separate laboratories and the choice of assay). Relative differences between strains in a given dataset are thus recognized to provide the most informative data (Ekland and Fidock, 2008). Consequently, we focus below primarily on relative differences between parasite lines expressing distinct *pfcr*t alleles.

For CQ, all recombinant lines expressing mutant *pfcr*t alleles (C8^{PH1}, C10^{PH2}, C12^{Cam734}, C4^{Dd2} and C6^{7G8}) had a statistically significant, 2.5 to 4.7-fold increase in mean IC₅₀ value relative to the isogenic recombinant C2^{GC03} line expressing the wild-type allele (mean IC₅₀ value of 14 nM) (Fig. 2A; Table S3). Of note, C10^{PH2} and C12^{Cam734} yielded CQ IC₅₀ values (53–54 nM) that were comparable in these assays to the recombinant C4^{Dd2} and C6^{7G8} parasites expressing the most globally prevalent mutant *pfcr*t alleles (Sidhu *et al.*, 2002). We note, however, that C4^{Dd2} and C6^{7G8} are no longer as CQ-resistant as when they were originally generated (in 2002) and characterized by several groups (Sidhu *et al.*, 2002; Lakshmanan *et al.*, 2005; Gligorijevic *et al.*, 2008). This has also been observed in an independent recent study that employed these lines (Hrycyna *et al.*, 2014). C4^{Dd2} and C6^{7G8} currently display CQ IC₅₀ values that are now 51% and 68% of the parental Dd2 and 7G8 lines respectively (Table S3), as compared to the initial report that documented corresponding values of 76% and 90%. These relative levels of CQR are illustrated for the original C4^{Dd2} and C6^{7G8} lines, alongside the now-attenuated lines, in Fig. 2A. Thus, our present data with the more recently generated PH1, PH2 and Cam734 *pfcr*t-expressing lines identify these as only modestly CQR, with relatively low CQ IC₅₀ values. Strikingly, the two C10^{PH2} clones demonstrated higher CQ mean IC₅₀ values as compared to C8^{PH1-II} parasites (53–54 nM compared to 35 nM). This finding implicates amino acid 72, which is the only polymorphism that distinguishes the PH2 and PH1 alleles (Table 1), as an important determinant of the degree of CQR.

Similar observations were made with the CQ metabolite monodesethyl-chloroquine (md-CQ), which yields much higher IC₅₀ values in CQ-resistant parasites, allowing for greater discrimination between resistant and sensitive lines (Sidhu *et al.*, 2002). C10^{PH2} and C12^{Cam734} clones all yielded mean IC₅₀ values (267–346 nM) that were comparable to C4^{Dd2} and C6^{7G8} (282 and 262 nM, respectively; Fig. 2B; Table S3). In comparison, the CQ-sensitive lines C2^{GC03} and GC03 showed mean IC₅₀ values of 17–22 nM. Of note, the mean md-CQ IC₅₀ value (164 nM) of the C8^{PH1} clone was considerably lower than the IC₅₀ values of the two C10^{PH2} lines (267 and 305 nM), again supporting a direct role for the C72S mutation in augmenting the degree of CQR.

Prior work has shown that CQ-resistant parasites can be chemosensitized to CQ and md-CQ by the resistance-reversing agent verapamil (VP) (Krogstad *et al.*, 1987; Martin *et al.*, 1987). The primary determinant of this reversibility trait has been mapped by quantitative trait loci analysis to mutant *pfcr1* (Patel *et al.*, 2010) and is more pronounced in the presence of the Dd2 allele as compared to the 7G8 allele (Mehlotra *et al.*, 2001; Sidhu *et al.*, 2002; Sa *et al.*, 2009). To assess VP reversibility in our recombinant lines, we performed drug assays in the presence or absence of 0.8 μM VP, and compared IC₅₀ values. Results showed a high degree of resistance reversal (74–85%) for C4^{Dd2} and the reference Dd2 line, and an intermediate degree (43–56%) for C6^{7G8} and 7G8 parasites (Fig. 2C). These values were significantly different from the CQ-sensitive C2^{GC03} line that along with GC03 showed no growth inhibition with this concentration of VP. Intriguingly, both C8^{PH1} and C10^{PH2} showed only a slight degree of reversal, which did not attain statistical significance. A similar lack of significant reversal was also observed with the metabolite md-CQ (Table S3). This agrees with an earlier report of Philippine isolates (Chen *et al.*, 2003) and is consistent with a recent study that associated PfCRT N75 (present in both haplotypes; Table 1) with minimal VP reversal (Sa *et al.*, 2009). We note that C12^{Cam734} parasites, which harbor the novel N75D mutation, were also subject to a significant degree (56–72%) of VP reversal of CQ and md-CQ resistance, at levels intermediate to the Dd2 and 7G8 alleles (Fig. 2C; Table S3).

We extended these studies to monodesethyl-amodiaquine (md-AQ), the clinically relevant metabolite of amodiaquine, a 4-aminoquinoline drug formerly used in monotherapy in many South American, African and Asian countries, including the Philippines (Sa *et al.*, 2009). This drug continues to be clinically important because of its incorporation into the widely used amodiaquine-artesunate combination (Wells *et al.*, 2009). Our studies reveal a substantial impact of both Philippine *pfcr1* alleles, as well as the Cam734 allele, on md-AQ responses (Fig. 2D), resulting in a 2.5 to 4-fold increase in IC₅₀ values compared to C2^{GC03}. Of all alleles tested, the smallest gain in md-AQ resistance was afforded by expression of the recombinant Dd2 allele that mediates relatively high-level CQR (see C4^{Dd2} responses in Fig. 2A, 2D). These data implicate an important role for the PfCRT mutations unique to the Philippine and Cambodian alleles in reducing parasite susceptibility to amodiaquine.

Importantly, every mutant *pfcr1* allele significantly increased susceptibility to the arylaminoalcohol drug lumefantrine (LMF; Fig. 2E), the partner drug comprising the most widely used artemisinin-based combination therapy (ACT), artemether-lumefantrine (Wells *et al.*, 2009). Mean IC₅₀ reductions were 37–47%, as compared to C2^{GC03} (Table S3). It is worth noting that a similar decrease in LMF IC₅₀ values in parasites expressing mutant *pfcr1*

as compared to isogenic parasites expressing the wild-type allele was earlier found to be associated with a significant reduction in the prevalence of mutant *pfcr*t in field isolates that recrudesced in patients treated with artemether-lumefantrine (Sisowath *et al.*, 2009). These findings support the therapeutic advantage of using LMF in areas of CQ-resistant malaria.

A similar trend was observed with the arylaminoalcohol mefloquine and the endoperoxide artemisinin. However the differences in IC₅₀ values did not attain statistical significance (Table S3). The PH1 and PH2 *pfcr*t alleles, but not Cam734, also significantly increased parasite susceptibility to quinine (Fig. 2F), a centuries-old drug used to treat severe malaria. Quinine resistance is known to be multifactorial, with quantitative trait loci analyses implicating mutant *pfcr*t and *pfmdr*1 as major determinants (Ferdig *et al.*, 2004; Sanchez *et al.*, 2011; Sanchez *et al.*, 2014). Our data support the hypothesis that the direction and magnitude of the effect of mutant *pfcr*t on quinine response depends on the genetic background and PfCRT haplotype (Cooper *et al.*, 2002; Sidhu *et al.*, 2002; Lakshmanan *et al.*, 2005; Cooper *et al.*, 2007). Finally, no differences were observed with piperazine (Table S3), an ACT partner drug that comprises two CQ 4-aminoquinoline rings tethered together with a spacer (Wells *et al.*, 2009), consistent with this drug being equally potent against parasites expressing wild-type or common mutant variants of *pfcr*t (Pascual *et al.*, 2013).

Reduced Chloroquine Accumulation Alone Does Not Account for Differences in the Degree of Chloroquine Resistance

Resistance to CQ has been associated with reduced drug accumulation in the DV and has previously been attributed to mutant PfCRT-mediated efflux of CQ from this acidic organelle (Krogstad *et al.*, 1987; Martin *et al.*, 1987; Valderramos and Fidock, 2006; Martin *et al.*, 2009). To investigate the influence of the Southeast Asian *pfcr*t alleles on CQ accumulation, we measured the kinetics of [³H]-CQ uptake in cultured parasites. The CQ-sensitive control lines GC03 and C2^{GC03} showed a rapid increase in CQ accumulation, as measured by a very high ratio (up to 2,300) of total intracellular CQ to extracellular CQ ([CQ]_{in}/[CQ]_{out}) (Fig. 3A). In contrast, the four *pfcr*t-variant recombinant lines (C4^{Dd2}, C8^{PH1-I}, C10^{PH2-I}, and C12^{Cam734-I}) accumulated minimal levels of CQ ([CQ]_{in}/[CQ]_{out}) values of ~200–300). Thus, these mutant PfCRT haplotypes, all expressed in the same genetic background, were associated with a very similar reduction of intracellular CQ levels (Fig. 3B), despite marked differences in their CQ IC₅₀ values (Fig. 2). These data lead us to suggest that reduced CQ accumulation might represent only one means by which mutant PfCRT dictates the degree of CQR.

PfCRT Haplotypes Influence Parasite Growth Rates in Co-Culture *in vitro* Competition Assays

To measure the extent to which *pfcr*t mutations influence the relative growth rates of asexual blood stage parasites, serving as a partial proxy for assessing fitness costs, we performed *in vitro* co-culture competition assays. In these assays, two lines were mixed in 1:1 ratios and the proportions of the individual *pfcr*t alleles were quantified by pyrosequencing every 4 days, on average, over a 2–3 month period. Data from these assays were converted using the ratio of the natural logs of the allelic frequencies, based on an assumption of exponential

growth, and were subjected to linear regression (Maree *et al.*, 2000; Mita *et al.*, 2004). Linear regression R^2 values were generally high (average 0.64, reflecting an acceptable goodness of fit; Table S5). In total, 34 co-culture assays were followed, yielding 518 measurements of *pfert* allelic frequencies over time (Table S5). From these measurements, we computed the mean relative growth rate value for each line, as detailed in the SI.

Results indicated that the isogenic clones C8^{PH1-II} and C10^{PH2-I}, expressing the PH1 and PH2 mutant *pfert* alleles respectively, each displayed reduced *in vitro* growth when independently co-cultured with C2^{GC03} parasites (Figs. 4; 5A). Of the two mutant *pfert* parasites, C10^{PH2-I} parasites showed the more substantial loss of relative growth rate *in vitro*. This finding was consistent with direct competition assays between C10^{PH2-I} and C8^{PH1-II}, which revealed reduced relative growth rates with the former (Table S5). This contrasted with CQ and md-CQ IC₅₀ values that were higher for C10^{PH2-I} (Fig. 2; Table S3). These results suggest a state of balanced polymorphisms whereby the PH1 allele could be predicted to fare better than the more unfit PH2 in mixed infections in the absence of CQ drug pressure, whereas the moderately more CQR PH2 allele could be more competitive in the presence of CQ and thus be retained in the population. Intriguingly, parallel studies with the CQ-resistant C12^{Cam734-I} line documented that these parasites were consistently as fit in terms of growth rates, if not slightly more so, when compared to C2^{GC03} *in vitro*. These data suggest that the Cam734 allele, harboring an unusually high number of point mutations (Table 1), has achieved a functional state that might reduce the fitness cost typically observed with mutant *pfert* alleles in endemic settings.

In contrast to Cam734, we observed a substantial reduction in relative growth rates associated with the Dd2 *pfert* allele when comparing C4^{Dd2} to C2^{GC03}, a finding consistent with reports of reduced fitness associated with this allele in African parasite populations (Kublin *et al.*, 2003; Mita *et al.*, 2003; Ord *et al.*, 2007). A more modest reduction in relative growth rates was observed with the 7G8 allele present in C6^{7G8}. The reduced relative growth rates of the Dd2 and 7G8 alleles is consistent with a recent report that also assessed these recombinant parasite lines in mixed culture experiments (Lewis *et al.*, 2014). In our studies, C6^{7G8} consistently outcompeted C4^{Dd2} in mixed cultures in four independent experiments (Fig. 4; Table S5). Importantly, when testing non-recombinant lines, we found the Dd2 line to have moderately increased growth rates relative to lines expressing wild-type *pfert* (GC03 and HB3), suggesting that Dd2 harbors additional compensatory mutations in its genome that corrected the growth defect associated with expression of its *pfert* allele. A summary of the influence of *pfert* alleles on *in vitro* growth rates in these co-competition assays is depicted in Fig. 5A. Independent support for these data came from measurements of *in vitro* growth rates for individual lines, which were monitored daily for seven days in three independent assays per line. Calculated multiplication rates per 48 hr revealed that in comparison to parasites expressing the *pfert* wild-type GC03 allele, parasites expressing the Cam734 allele showed an equivalent growth rate, whereas parasites expressing the PH2, Dd2 and 7G8 alleles displayed slower rates of growth (Fig. S1).

Discussion

Genome-wide studies of *P. falciparum* populations have demonstrated a remarkable degree of recent evolution in the *pfcr* coding sequence, beginning with several independent origins of variant alleles. These alleles have spread across malaria-endemic regions as selective sweeps driven by intense drug pressure (Wootton *et al.*, 2002; Kidgell *et al.*, 2006; Volkman *et al.*, 2007; Mu *et al.*, 2010). Primary origins have been localized to South America and Papua New Guinea (independent sources of the 7G8 variant haplotype), the Philippines (PH1 and PH2), the Thai-Cambodian border (Dd2), and most likely Cambodia (Cam734; Fig. 5B) (Ecker *et al.*, 2012). CQR in Africa resulted from the introduction of variant *pfcr* of Southeast Asian origin (Ariey *et al.*, 2006), whose insidious impact on malaria rates was highlighted by reports from Senegal showing nearly a six-fold increase in malarial deaths following the arrival of resistant strains (Snow *et al.*, 2001; Trape, 2001). Our study of geographically distinct PfCRT haplotypes reveals an intricate balance between CQR and parasite growth rates, which provide an *in vitro* proxy of fitness. Our data also highlight the emergence (in Cambodia) of the Cam734 allele (Durrand *et al.*, 2004), which appears to have succeeded in mediating a moderate degree of resistance while concurrently maintaining *in vitro* growth rates at least as good if not better than wild-type *pfcr* (Fig. 5C). Notably, Cambodia is a known hotbed of multidrug resistance, beginning with CQ and pyrimethamine-sulfadoxine and more recently with emerging resistance to artemisinin derivatives (Dondorp *et al.*, 2011; Ariey *et al.*, 2014; Straimer *et al.*, 2015). We ascribe the lack of an observable growth rate defect in the Cam734 allele to its complex set of mutations (Table 1), which include the L148I, I194T and T333S mutations not present in other parasites studied herein. We posit that these mutations have evolved to compensate for a loss of fitness bestowed by an initial set of mutations that were sufficient to confer CQR, including but not limited to K76T (Lakshmanan *et al.*, 2005). The ability of *Plasmodium* parasites to acquire a set of mutations simultaneously conferring CQR and enhanced fitness has previously been documented in murine studies, in which CQ-resistant *P. chabaudi* parasites outgrew their sensitive counterparts, even when mice were inoculated with a nine-fold excess of sensitive parasites (Rosario *et al.*, 1978). Recent metabolomic studies show that the widespread Dd2 and 7G8 alleles cause increased levels of intracellular peptides in asexual blood stage parasites, presumably stemming from impaired hemoglobin digestion that restricts the supply of amino acids required for parasite proliferation (Lewis *et al.*, 2014). These recent findings provide a potential mechanistic explanation for the fitness costs observed with the Dd2 and 7G8 haplotypes. Further studies are required to assess whether the Cam734 allele corrects this abnormal accumulation of hemoglobin-derived peptides.

Our findings also illustrate a singular impact of PfCRT residue 72, which is the sole sequence distinction between the Philippine PH1 allele that mediates marginal CQR and the PH2 allele that is moderately more resistant (Fig. 5C). This residue is also associated with differences in relative growth rates *in vitro* (Fig. 4; 5A). How this single amino acid difference might impact protein conformation, stability or post-translational modifications remains to be determined. During these experiments, we isolated a separate recombinant C8^{PH1} parasite, referred to as C8^{PH1-II}, which underwent a spontaneous loss of the N326D mutation during extended culture. This reversion back to the wild-type residue was

associated with an increased rate of parasite propagation and a loss of CQR. The reversion to a CQS phenotype by the N326D mutation is consistent with a recent report that showed a lack of CQ transport in *Xenopus* oocyte-based heterologous expression assays, in contrast to the PH1 PfCRT variant that showed CQ transport behavior (Summers *et al.*, 2014). These data highlight a requirement for multiple PfCRT mutations in producing the CQR phenotype, arguing against the notion that CQR results solely from the K76T mutation and that the other mutations in this protein compensate solely for loss of function. This conclusion is supported by transport studies in an oocyte expression system, which provided evidence that the K76T mutation needed to be accompanied by either the N75E (Southeast Asian PfCRT variants) or the N326D mutation (Latin American and oceanic PfCRT variants) to attain a CQ transport function (Summers *et al.*, 2014). While two mutational changes sufficed for a basal CQ transport activity, additional mutations were required for full activity. The order in which these mutations were added was important to avoid reductions in CQ transport activity (Summers *et al.*, 2014).

Epidemiological studies have found that some parasites harboring the PfCRT K76T mutation have low to moderate CQ IC₅₀ values that do not meet the standard definition of CQR and in some instances are similar to values observed with CQ-sensitive parasites, for example Cambodian isolates harboring the Cam734 allele (Durrand *et al.*, 2004). Studies are ongoing to dissect the role of the PfCRT SNPs that are unique to this haplotype. Furthermore, *in vitro* selection studies have shown that parasites harboring the Dd2 *pfcr*t allele acquired a C101F mutation that resulted in a loss of CQR despite the presence of K76T (Eastman *et al.*, 2011). Thus, while K76T continues to be an important molecular marker of CQR, recent evidence suggests that additional PfCRT SNPs can substantially modify the CQ response, in some cases causing an attenuation or loss of the resistance phenotype. We also note that while PfCRT is widely recognized to be the primary mediator of CQR, several studies point to a requirement for secondary determinants to augment CQR, including *pfmdr1* (Sidhu *et al.*, 2002; Sa *et al.*, 2009; Patel *et al.*, 2010; Valderramos *et al.*, 2010; Gaviria *et al.*, 2013).

In our mixed infection studies with isogenic *pfcr*t-modified clones we also observed that parasites displayed the greatest loss of asexual blood stage growth when expressing the Dd2 allele, consistent with its progressive disappearance from high-transmission African settings in the absence of CQ pressure (Mita *et al.*, 2003; Ord *et al.*, 2007; Mwai *et al.*, 2009; Laufer *et al.*, 2010; Frosch *et al.*, 2011). This contrasts with the situation in Southeast Asia where the Dd2 allele remains at high frequencies in the absence of CQ pressure. Fewer mixed infections in Asia compared to Africa likely result in less opportunity for competition with the wild-type allele. The lesser fitness cost observed with the 7G8 allele, which is prevalent in South America and the Pacific region, is concordant with studies from these regions showing the continued presence of mutant *pfcr*t despite minimal CQ use in recent decades to treat *P. falciparum* malaria (Mu *et al.*, 2010). We note that modest selective pressure on mutant *pfcr*t may also have come from the use of CQ to treat *P. vivax* infections, which are common outside of Africa (Price *et al.*, 2007). Overall, our *in vitro* mixed competition relative growth rate data would suggest that the idea of reintroducing CQ into regions where prolonged drug removal has led to the near disappearance of resistant strains (Juliano *et al.*,

2007; Laufer *et al.*, 2010) is suitable only in areas harboring mutant *pfcr*t alleles such as Dd2 that cause reduced fitness, and would be less applicable to regions harboring relatively “fit” alleles such as Cam734.

The loss of CQ efficacy across the globe, followed by a short-lived dependence on the sulfadoxine-pyrimethamine antifolates, has resulted in recent years in the global adoption of ACTs (White, 2008; Eastman and Fidock, 2009). Notably, our study shows that all mutant *pfcr*t alleles tested herein, including the two that are most prevalent (Dd2 and 7G8), increase parasite susceptibility to lumefantrine. This is particularly significant as this drug, partnered with artemether (CoArtem®), is globally the most widely used antimalarial (Wells *et al.*, 2009). While the fold change in lumefantrine IC50 values is relatively low (2-fold; Fig. 2E; Table S3), we note that a clinical trial from Tanzania observed significant selection against mutant *pfcr*t parasites harboring the ⁷²CVIET⁷⁶ PfCRT haplotype following artemether-lumefantrine treatment (Sisowath *et al.*, 2009).

Amodiaquine-artesunate is another ACT that is often used in Africa (Olliaro and Mussano, 2000). We observed significantly reduced parasite susceptibility to the amodiaquine metabolite md-AQ with every tested mutant *pfcr*t allele. This includes both Philippine alleles, obtained from a country where amodiaquine has been used as an antimalarial treatment for over 40 years (Sa *et al.*, 2009). We thus posit that amodiaquine could have been a major contributor to the emergence and/or maintenance of the PH1 and PH2 alleles. An important role for amodiaquine in driving the spread of the ⁷²SVMNT⁷⁶ PfCRT haplotype (present in 7G8) has recently been proposed based on studies of parasites from South America and Asia (Sa *et al.*, 2009; Beshir *et al.*, 2010), and amodiaquine pressure could conceivably account for the apparent recent spread of this haplotype into Africa and in India (Alifrangis *et al.*, 2006; Gama *et al.*, 2010; Mixson-Hayden *et al.*, 2010). In addition, as with many Asian countries, CQ has continued to be used to treat patients infected with *P. vivax*, thus sustaining local CQ pressure that could influence the course of mixed infections of *P. vivax* and *P. falciparum*. Overall, our data support the use of artemether-lumefantrine in preference to amodiaquine-artesunate to treat CQ-resistant malaria. We also found no evident effect of mutant *pfcr*t on the efficacy of piperazine, an ACT partner drug with excellent clinical efficacy and post-treatment prophylactic activity (Wells *et al.*, 2009).

Our mechanistic investigations into CQR provide evidence that mutant PfCRT-mediated CQR can be phenotypically distinguished from reduced intracellular CQ accumulation, as also noted by a previous study (Sanchez *et al.*, 2011). This was particularly evident with C8^{PH1} parasites that, despite having only a nominal degree of CQR, displayed kinetics of intracellular CQ accumulation that paralleled the other, more CQ-resistant parasites. In contrast, CQ-sensitive parasites showed 7–10 fold higher levels of CQ accumulation (Fig. 4). Thus, all mutant PfCRT variants shared an ability to reduce CQ accumulation. This study agrees with recent evidence that reduced CQ accumulation is not the sole cause of CQR (Cabrera *et al.*, 2009; Sanchez *et al.*, 2011; Baro *et al.*, 2013). We posit that mutant PfCRT generally reduces CQ accumulation, and that this is an essential feature of CQR, but that various PfCRT haplotypes differ in a second respect that further contributes to the CQR phenotype. One possibility is that the higher degree of CQR reflects varying degrees to which PfCRT functions to also reduce the cellular toxicity associated with CQ action,

possibly by negating the effect of CQ on preventing the buildup of reactive heme-iron or oxygen species liberated following hemoglobin proteolysis. Another possibility is that the drug competes with a yet to be identified physiological substrate for transport via PfCRT and that this competition impacts on the natural function of the transporter. In this context it is interesting to note that PfCRT appears capable of simultaneously accepting different substrates at distinct but antagonistically interacting binding sites (Bellanca *et al.*, 2014). Binding of two different substrates might result in an inactive transporter or one with substantially reduced activity, depending on the nature of the substrates bound (Bellanca *et al.*, 2014). How a geographic PfCRT variant copes with its drug and physiological transport functions is likely determined by its specific amino acid substitutions. Further dissection of the biochemical parameters associated with heme detoxification and CQ action can now be achieved using the series of isogenic *pfert*-modified lines described herein.

Experimental Procedures

Parasite Culture, Transfection, and Selection and Characterization of Integrant Clones

Parasites were cultured at 37°C in human red blood cells in Albumax-containing culture medium, as described (Fidock *et al.*, 1998). Isogenic lines expressing variant *pfert* alleles were generated following transfection of the C1^{GC03} clone (Sidhu *et al.*, 2002). This clone was previously generated from GC03 (a progeny of the HB3×Dd2 genetic cross (Wellems *et al.*, 1990)), and expresses wild-type *pfert* from a recombinant locus lacking introns 2–12 (Fig. 1). C1^{GC03} parasites were propagated to ~8% ring stage parasitemia and electroporated with 50 µg of plasmid (pBSD-crt-PH1^{Py3'}, pBSD-crt-PH2^{Py3'} or pBSD-crt-Cam734^{Py3'}; Table 1; Fig. 1). Transformed parasites were selected using 2.5 nM WR99210 (Jacobus Pharmaceuticals; Princeton, NJ) and 2.5 µM Blasticidin HCl (Invitrogen). Successfully transfected parasites were detectable in culture 2–3 weeks post-transfection and cloned by limiting dilution once plasmid integration was detected. Details of plasmid construction, and of the molecular characterization of parasite lines are provided in the SI (primers listed in Table S1). *pfert*-modified transgenic lines will be made available upon request and are being deposited in the MR4 Malaria Reagent Repository.

In vitro Drug Susceptibility Assays

Parasite susceptibilities to antimalarial drugs were assessed *in vitro* as described (Fidock *et al.*, 1998) using 72 hr [³H]-hypoxanthine assays (see SI). IC₅₀ values were calculated by non-linear extrapolation. Statistical analyses employed Mann-Whitney *U* tests.

In vitro Mixed Culture Competition Assays, Pyrosequencing and Determination of Relative Growth Rate Values

For growth competition assays, two parasite lines were mixed 1:1 and seeded in duplicate or triplicate at an initial parasitemia of 0.6% ring stage parasites, in drug-free medium. Parasitemias were maintained between 0.3% and 8% to assure optimal growth conditions. Two to six separate competition assays were performed for each drug-free mixture and each assay was monitored for an average of 66 days (range 43–90; Fig. 4; Table S5). 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 four days (range 2–9) and DNA was

extracted using DNeasy Blood & Tissue Kits (Qiagen). The DNA was then used for ratiometric determination of individual allele frequencies in these mixed cultures by pyrosequencing of codon position 72 or 76 (detailed in the SI). To calculate the relative growth rates of individual parasite lines, the relative proportion of the two distinct *pfcr* alleles (whose values were always between 0 and 1, inclusive) were natural log-transformed and linear regression was applied to estimate the relative growth rate value, as detailed in the SI. These values, along with the calculated SEM and R^2 values, are listed in Table S5.

Chloroquine Accumulation Assays

These were performed, as previously described (Sanchez *et al.*, 2003), using magnet-purified, sorbitol-synchronized trophozoites (detailed in SI). The amount of accumulated intracellular [^3H]-CQ was calculated as the ratio of $[\text{CQ}_{\text{in}}]/[\text{CQ}_{\text{out}}]$, normalized to 1×10^6 infected erythrocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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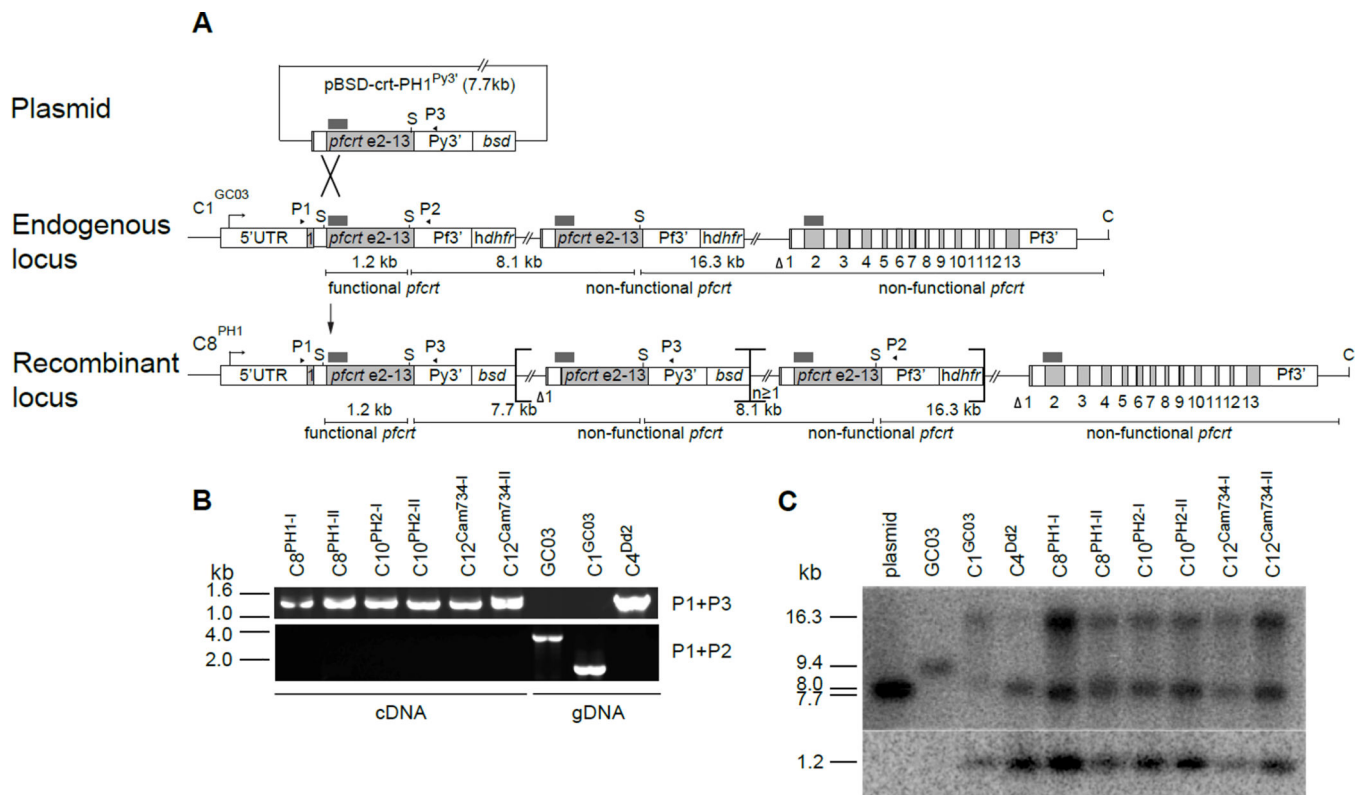


Fig. 1. *pfert* allelic exchange strategy and molecular characterization of clones

(A) Schematic representation of single-site crossover between a pBSD-based *pfert* transfection plasmid and the functional *pfert* locus of C1^{GC03}, leading to expression of a recombinant allele (PH1, PH2, or Cam734; see Table 1) from the endogenous full-length promoter. The diagram illustrates transfection of the CQ-sensitive C1^{GC03} clone with pBSD-crt-PH1^{Py3'}. This construct contained a *pfert* sequence with a deletion in exon 1, no introns between exons 2–13 and a downstream 0.7 kb 3' UTR sequence from the *P. yoelii* ortholog *pycrt*. Homologous recombination upstream of codon positions 74–76 resulted in generation of a functional *pfert* allele containing all the point mutations from the mutant PH1 allele, under the control of *pfert* 5' UTR and *pycrt* 3' UTR regulatory elements. Downstream remnant *pfert* fragments were truncated in exon 1, had a 5' in-frame stop codon and lacked a promoter. (B) PCR-based analysis of the recombinant clones and parental lines (primer positions illustrated in Fig. 1A). Parasite strain and primer details are provided in Table 1 and Table S1, respectively. (C) Southern blot hybridization of gDNA digested with SalI and ClaI and subsequently probed with a *pfert* fragment from exon 2. The positions of the restriction sites and exon 2-specific probe are indicated in Fig. 1A. Panels B and C include the clone C8^{PH1-I}, which was subsequently found to have undergone a spontaneous mutation in codon 326 and was removed from further analysis.

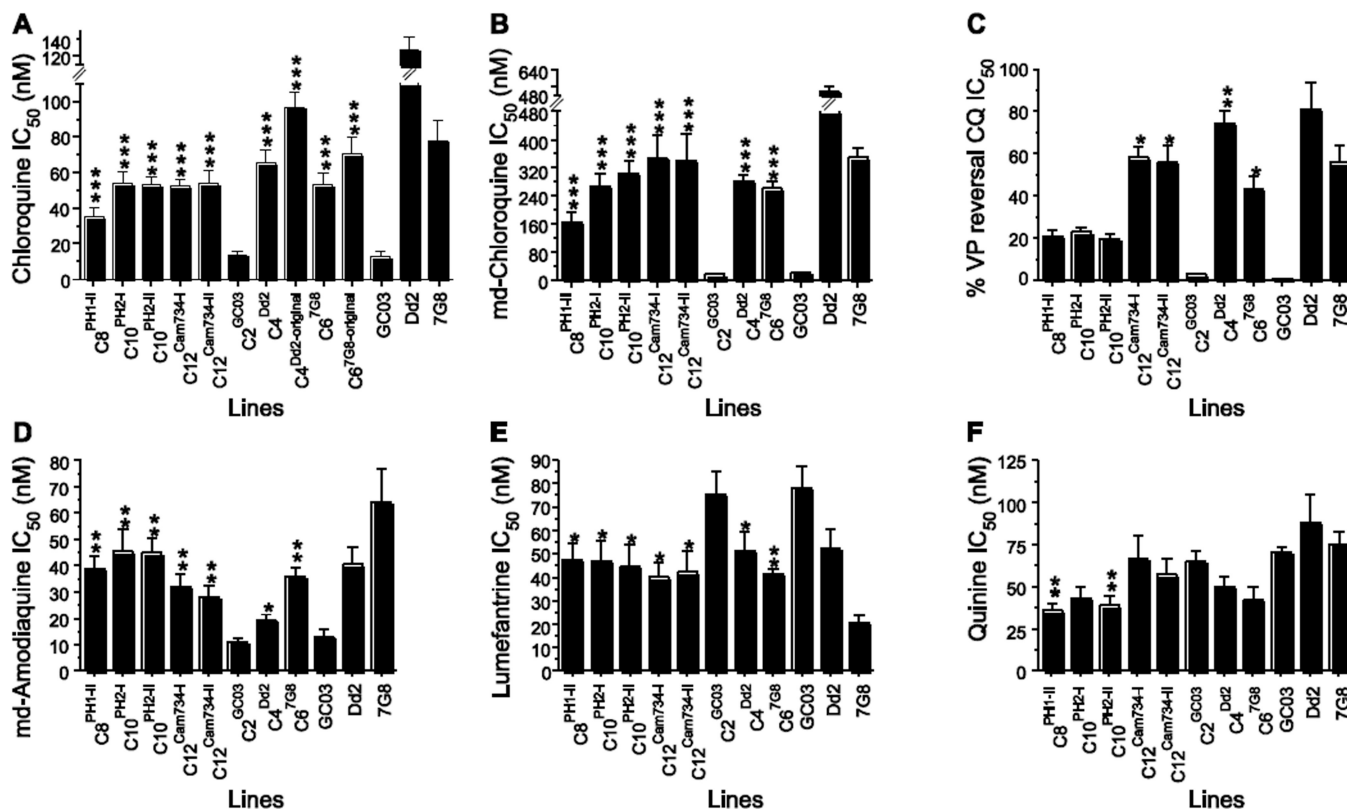


Fig. 2. Susceptibility of *pfert*-modified clones to selected antimalarial drugs

Mean IC₅₀ values ± SEM for the indicated parasite strains subjected to clinically significant antimalarials, as measured *in vitro* using [³H]-hypoxanthine incorporation assays. We note that the CQ values, both for resistant and sensitive strains, are markedly lower than earlier reports (Sidhu *et al.*, 2002; Lakshmanan *et al.*, 2005; Valderramos *et al.*, 2010), and coincide with our reducing the HEPES concentration from the earlier 50 mM to the current 25 mM (see SI). CQ values for the original C4^{Dd2} and C6^{7G8} lines, presented as a proportion of their IC₅₀ values of the reference Dd2 and 7G8 lines, are included to illustrate the attenuation of the CQR phenotypes of these lines over time. The C4^{Dd2}-original CQ mean±SEM IC₅₀ value of 96.8±8.7 nM is comparable to the values of 91.8±10.7 nM and 100.3±15.5 nM recently reported with two *pfert*-modified GC03 clones engineered to express the Dd2 allele (i.e. analogous to C4^{Dd2}) using customized zinc-finger nucleases (Straimer *et al.*, 2002). VP reversal was calculated as the IC₅₀ of CQ + 0.8 μM VP divided by the IC₅₀ of CQ. VP reversal values for CQ, md-CQ and md-ADQ are provided in Table S4. Mann-Whitney *U* tests were used to assess for statistically significant differences between a recombinant line expressing mutant *pfert* and the CQ-sensitive line C2^{GC03} expressing wild-type *pfert*. **P*<0.05; ***P*<0.01; ****P*<0.001. IC₅₀ and IC₉₀ values, numbers of assays, and tests for significance are reported in Table S3.

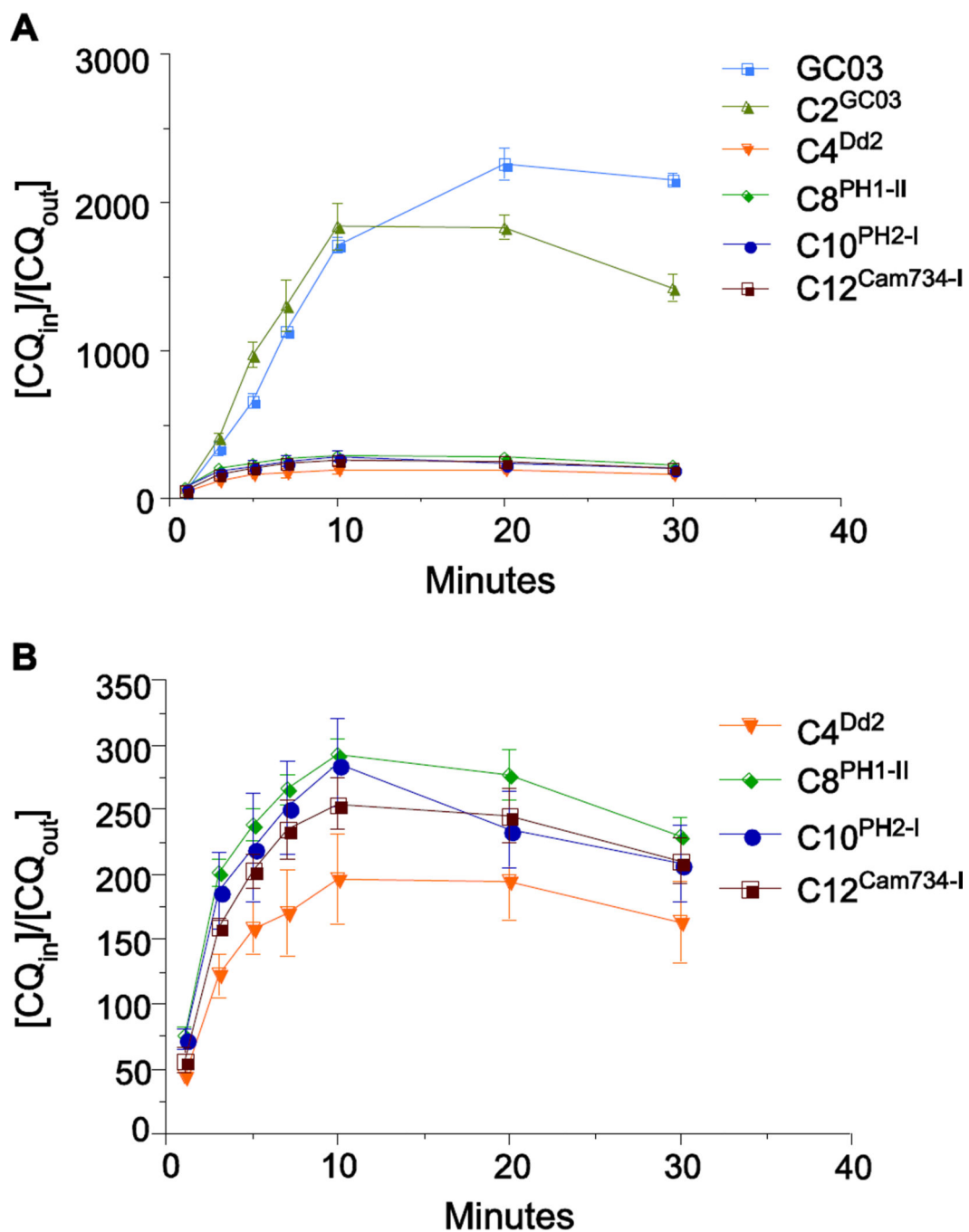


Fig. 3. Chloroquine accumulation of *pfert*-modified parasite lines

$[^3\text{H}]$ -CQ accumulation is represented as the ratio of the total intracellular CQ to extracellular CQ ($[\text{CQ}_{\text{in}}]/[\text{CQ}_{\text{out}}]$). Values represent the mean \pm SEM determined from three independent experiments performed in duplicate. Data are shown in (A) for the full set of *pfert*-modified lines and the CQ-sensitive control GC03, and in (B) exclusively for the lines expressing mutant *pfert* (note the reduced Y-axis scale).

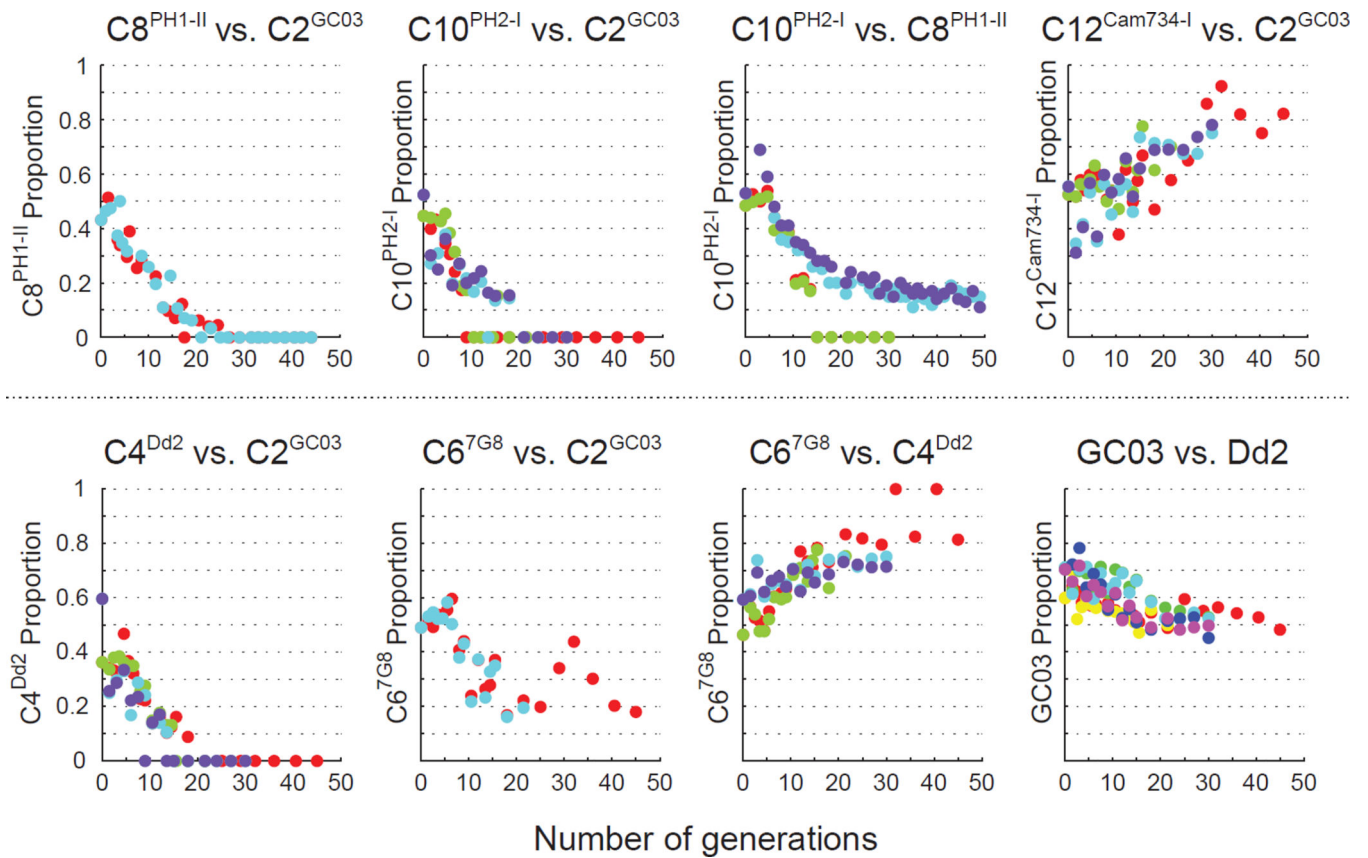


Fig. 4. Relative growth rate plots for mixed competition assays between *pfprt*-modified lines Parasite cultures were initiated at ~1:1 ratios and allelic proportions were measured over time for up to 45 generations by pyrosequencing. The Y-axis illustrates the proportion of the first listed allele comprising the mixed cultures. Each color represents a separate assay. Data are collectively summarized in Fig. 5A and Table S5.

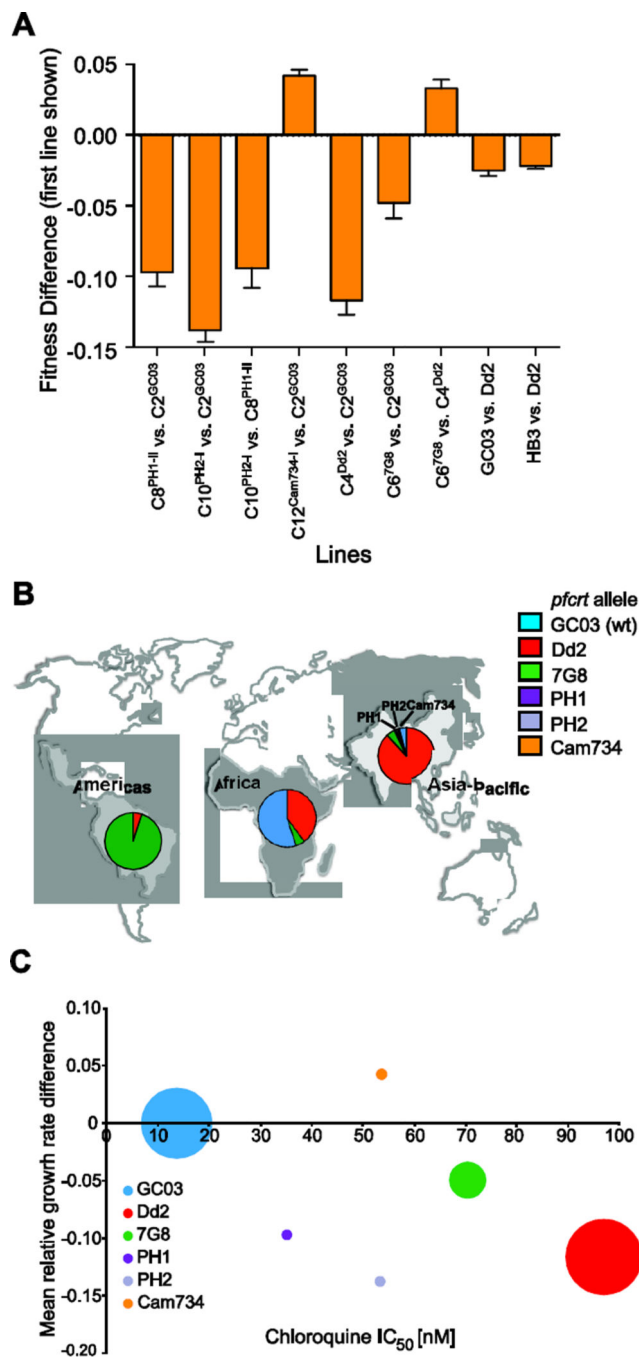


Fig. 5. Influence of *pfprt* alleles on relative growth rates and chloroquine resistance
 (A) Relative growth rate values for the individual *pfprt*-modified parasite lines were measured following pyrosequencing-based assessment of changes in *pfprt* allele frequencies in mixed cultures maintained for up to 45 generations. For each combination, 1–6 independent competition assays were performed in duplicate (summarized in Table S5; data plotted in Fig. 4). The histogram depicts the relative growth rate of the first line compared to the second, e.g. for C8^{PH1-II} vs. C2^{GC03} the negative value reflects the reduced relative growth rate of C8^{PH1-II}. (B) Distribution of various *pfprt* alleles in the major malaria-

endemic regions. (C) Mean relative growth rate differences between wild-type and mutant *pfcr* alleles (in the GC03 background) presented as a function of the effect of these alleles on CQ IC₅₀ values (based on data presented in Tables S3 and S5 and depicted in Figs. 2 and 5A). Circle size indicates the estimated worldwide frequency of the *pfcr* allele, approximated from literature reports and database summaries on the number of clinical cases and the distribution of *pfcr* alleles (see Table S6)

Table 1

Transformation status and PfCRT haplotype of recombinant and wild-type lines.

Clone	Parent	Transfection plasmid*	Functional PfCRT haplotype															
			72	74	75	76	144	148	160	194	220	271	326	333	356	371		
GC03	HB3 × Dd2	-	C	M	N	K	A	L	L	L	I	A	Q	N	T	I	R	
C1 ^{GC03}	GC03	phDHER-crt-GC03 ^{PI3'}	C	M	N	K	A	L	L	L	I	A	Q	N	T	I	R	
C8 ^{PHI-II}	C1 ^{GC03}	pBSD-crt-PHI ^{Py3'}	C	M	N	T	T	L	Y	I	A	Q	D	T	I	R		
C10 ^{PH2-III}	C1 ^{GC03}	pBSD-crt-PH2 ^{Py3'}	S	M	N	T	T	L	Y	I	A	Q	D	T	I	R		
C12 ^{Cam734-III}	C1 ^{GC03}	pBSD-crt-Cam734 ^{Py3'}	C	I	D	T	F	I	L	T	S	E	N	S	I	R		
C2 ^{GC03}	C1 ^{GC03}	pBSD-crt-Dd2 ^{PI3'}	C	M	N	K	A	L	L	L	I	A	Q	N	T	I	R	
C4 ^{Dd2}	C1 ^{GC03}	pBSD-crt-Dd2 ^{Py3'}	C	I	E	T	A	L	L	L	I	S	E	S	T	I		
C6 ^{G8}	C1 ^{GC03}	pBSD-crt-7G8 ^{Py3'}	S	M	N	T	A	L	L	L	I	S	Q	D	T	L	R	
HB3	-	-	C	M	N	K	A	L	L	L	I	E	Q	N	T	I	R	
Dd2	-	-	C	I	E	T	A	L	L	L	I	S	E	S	T	T	I	
7G8	-	-	S	M	N	T	A	L	L	L	I	S	Q	D	T	L	R	

Plasmids harboring different *pfcr* allelic sequences were transfected into CQ-sensitive C1^{GC03} parasites to generate the recombinant mutant and control lines. Grey shading indicates residues that differ from the wild-type sequence.