January 2009

In vivo selection of Plasmodium falciparum parasites carrying the chloroquine-susceptible pfcrt K76 allele after treatment with artemether-lumefantrine in Africa

C. Sisowath
Karolinska Institutet

I. Petersen
Columbia University

M.I. Veiga
Karolinska Institutet

A. Martensson
Karolinska Institutet

Zul Premji
Aga Khan University, zul.premji@aku.edu

See next page for additional authors

Authors
C. Sisowath, I. Petersen, M.I. Veiga, A. Martensson, Zul Premji, A. Bjorkman, D.A. Fidock, and J.P. Gil

Published in final edited form as:
J Infect Dis. 2009 March 1; 199(5): 750–757. doi:10.1086/596738.

In Vivo Selection of *Plasmodium falciparum* Parasites Carrying the Chloroquine-Susceptible *pfcrt* K76 Allele after Treatment with Artemether-Lumefantrine in Africa

Christin Sisowath[1], **Ines Petersen**[5], **M. Isabel Veiga**[1,3], **Andreas Mårtensson**[1,2], **Zul Premji**[4], **Anders Björkman**[1], **David A. Fidock**[5,6], and **José P. Gil**[1,3]

[1]Infectious Diseases Unit, Department of Medicine, Karolinska Institutet, Stockholm, Sweden

[2]Division of Internal Health, Department of Public Health Sciences, Karolinska Institutet, Stockholm, Sweden

[3]Pharmacogentics and Drug Resistance Group, Centre of Molecular and Structural Biomedicine/ Institute of Biotechnology and Bioengineering, University of Algarve, Gambelas, Portugal

[4]Department of Parasitology, Muhimbili University of Health and Allied Sciences, Dar Es Salaam, Tanzania

[5]Department of Microbiology, College of Physicians and Surgeons, Columbia University, New York, New York

[6]Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, New York

Abstract

Background—Artemether-lumefantrine (AL) is a major and highly effective artemisinin-based combination therapy that is becoming increasingly important as a new first-line therapy against *Plasmodium falciparum* malaria. However, recrudescences occurring after AL treatment have been reported. Identification of drug-specific parasite determinants that contribute to treatment failures will provide important tools for the detection and surveillance of AL resistance.

Method—The findings from a 42-day follow-up efficacy trial in Tanzania that compared AL with sulfadoxine-pyrimethamine (SP) were analyzed to identify candidate markers for lumefantrine tolerance/resistance in the chloroquine resistance transporter gene *(pfcrt)* and multidrug resistance gene 1 *(pfmdr1)*. The findings were corroborated in vitro with genetically modified isogenic *P. falciparum* parasite lines.

Results—Treatment with AL selected for the chloroquine-susceptible *pfcrt* K76 allele ($P < .0001$) and, to a lesser extent, the *pfmdr1* N86 ($P = .048$) allele among recurrent infections. These genotypes were not selected during SP treatment. No *pfmdr1* gene amplifications were observed. Isogenic *pfcrt*-modified parasite lines demonstrated a 2-fold increase in susceptibility to lumefantrine, which was directly attributable to the K76T mutation.

Reprints or correspondence: Dr. Christin Sisowath, Malaria Research, Scheele-lab B2, Retzius väg 10 pl. 5, 171 77 Stockholm, Sweden (E-mail: christin.sisowath@ki.se).

Potential conflicts of interest: none reported.

Presented in part: Molecular Approaches to Malaria–2008, Lorne, Victoria, Australia, 3–7 February 2008 (abstract 132).

Conclusions—Our findings suggest that the *pfcrt* K76T mutation is a drug-specific contributor to enhanced *P. falciparum* susceptibility to lumefantrine in vivo and in vitro, and they highlight the benefit of using AL in areas affected by chloroquine-resistant *P. falciparum* malaria.

The development and spread of *Plasmodium falciparum* antimalarial drug resistance has spurred a global change in policy from the use of the former first-line antimalarials chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) to the use of artemisinin-based combination therapies (ACTs). In 2006, Tanzania changed its drug policy to the use of artemether-lumefantrine (AL; Coartem [Novartis]), which is currently the most favored ACT used in Africa.

AL is highly effective on the African continent [1–3]. Nevertheless, AL has been associated with a relatively high frequency of reinfection events during the post-treatment period, when lumefantrine, the longer-lasting partner drug (with a plasma half-life of 4–5 days vs. 3–11 h for artemether), remains in the blood in subtherapeutic concentrations. During this period, selection of less-susceptible parasites (which have the ability to cause infection in the presence of plasma drug concentrations higher than those associated with susceptible parasites causing infection) may occur.

One gene that has been found to influence susceptibility to lumefantrine is the *P. falciparum* multidrug resistance gene 1 (*pfmdr1*), which encodes a transporter located on the digestive vacuole (DV) membrane of the erythrocytic stages. Allelic exchange experiments with culture-adapted lines, as well as field studies of clinical isolates, have shown that *pfmdr1* single-nucleotide polymorphisms (SNPs) influence the in vitro response to various antimalarial drugs, such as the artemisinin derivatives and the arylaminoalcohols lumefantrine, mefloquine (MQ), and halofantrine (HF) [4–7]. An increased *pfmdr1* copy number has also been associated with treatment failure after short 4-dose AL therapy [8], as well as after MQ or artesunate-MQ therapy [9]. So far, these associations have been observed only in Southeast Asia. However, an increased number of *pfmdr1* copies has been found in a few patient samples in Gabon, subsequent to a trial with low-dose MQ treatment, thereby suggesting the potential of selection of multicopy *pfmdr1* in this region [10]. Hence, the *pfmdr1* copy number may also become important on the African continent after wide-scale deployment of AL.

Our previous studies in Zanzibar showed that treatment with AL selected for reinfecting parasites harboring *pfmdr1* alleles encoding the N86, 184F, and D1246 residues [11,12]. These findings are consistent with reports from other East African field sites [13,14] and suggest the selection of lumefantrine-tolerant parasites, which may constitute a first step toward full resistance and associated clinical failure [15]. Nonetheless, it remains unclear whether this selection is the result of specific influences on the drug target or is caused by nonspecific selection of parasites that are generally more fit and able to thrive in the presence of environmental stressors. This is an important distinction, considering that mutations at codons 1034, 1042, and 1246 in *pfmdr1* have been reported to incur fitness costs in vitro [16].

Another question arising from our previous studies is the role of the chloroquine resistance transporter gene *(pfcrt)*, which encodes a transmembrane protein that localizes to the membrane of the DV and is a key determinant of the chloroquine response of *P. falciparum* [17]. The *pfcrt* K76T mutation has been consistently associated with CQ resistance in vitro and in vivo [18–20]. This putative transporter also appears to influence the parasite response to arylaminoalcohols. Accordingly, mutations at the *pfcrt* amino acid 76 position have been suggested to play a role in MQ susceptibility [19–21]. In addition, in vitro selection of HF resistance has been associated with a novel *pfcrt* S163R mutation, which also has been shown to affect the in vitro response to MQ [22]. In light of these data, we hypothesized that *pfcrt* SNPs might also influence susceptibility to lumefantrine.

To evaluate the influence of *pfmdr1* and *pfcrt* mutations on the mechanism of resistance to lumefantrine, we searched for selection of these mutations in samples collected during a clinical trial in Fukayosi, Tanzania, in which children with uncomplicated *P. falciparum* malaria were treated with AL or SP [2]. SP inhibits the synthesis of folate by targeting the dihydropteroate synthetase and dihydrofolate reductase enzymes, respectively. This treatment arm therefore enabled us to test whether any evidence of selection for *pfcrt* or *pfmdr1* mutations in the AL arm was drug dependent and was not a result of general fitness issues associated with the parasite response to any antimalarial drug treatment. Furthermore, the availability of isogenic parasites resulting from allelic exchange experiments at *pfcrt* amino acid position 76 [19,20] allowed us to evaluate the specific influence of mutant *pfcrt* alleles (in particular, the K76T mutation) on the response of *P. falciparum* to lumefantrine.

SUBJECTS, MATERIALS, AND METHODS

Subjects

The study was conducted in Fukayosi, Bagamoyo District, Tanzania, during April–July 2004, as reported elsewhere [2]. In brief, 106 children with uncomplicated *P. falciparum* malaria were enrolled and randomly allocated to receive either SP (Fansidar; Roche) or AL (Coartem). Both drugs were administered according to body weight. Children who were allocated to receive SP and who had body weights categorized as 5–10 kg, 11–20 kg, and 21–30 kg were given a single dose of sulfadoxine/pyrimethamine of 250 mg/12.5 mg, 500 mg/25 mg, and 750 mg/37.5 mg, respectively, whereas children who were allocated to receive AL and who had body weights categorized as 5–14 kg, 15–24 kg, and 25–34 kg were given a twice-daily dose of artemether/lumefantrine of 20 mg/120 mg, 40 mg/240 mg, and 60 mg/360 mg, respectively, for 3 days. After enrollment in the study and initiation of treatment, the children were checked routinely on days 1, 2, 3, 7, 14, 21, 28, 35, and 42 or on any day of recurrent illness occurring during the 42-day follow-up. Blood samples were collected on filter paper (3MM; Whatman) for molecular analysis. Written informed consent was obtained from the parents or guardians of the children. The study was approved by the ethics committees of the Muhimbili University College of Health and Allied Sciences (Dar Es Salaam, Tanzania) and Karolinska Institutet (Stockholm, Sweden).

Molecular analysis

DNA was extracted from blood samples on filter paper by use of the ABI Prism 6100 Nucleic Acid PrepStation (Applied Biosystems). The status of recurrent infections (recrudescence or reinfection) was available from a previous study [2] that used stepwise genotyping of the polymorphic genetic markers *P. falciparum* merozoite surface protein *(pfmsp)*–2 and –1 to classify treatment outcomes. In the present study, we analyzed the first baseline day (D_0) and the initial day of recurrent infection (R_0).

Mutations in *pfmdr1* and *pfcrt* were investigated to explore their association with AL tolerance. All infections observed at baseline were examined, as were all infections that recurred after AL treatment. The *pfmdr1* N86Y and D1246Y SNPs, as well as the *pfcrt* K76T and S163R SNPs, were analyzed by polymerase chain reaction (PCR) restriction fragment–length polymorphism (RFLP) analysis, as described elsewhere [12,23]. The *pfmdr1* Y184F SNP was assayed using pyrosequencing (Pyro-Gold Reagents PSQ 96MA and PyroMark MD System; Biotage AB) with the primer 5'-CCAGTTCCTTTTTAGGTT-3'. The *pfmdr1* S1034C and N1042D SNPs were examined through direct sequencing of PCR products (Macrogen). For infections recurring after SP treatment, only the *pfmdr1* N86Y and *pfcrt*K76T mutations were assessed. The *pfmdr1* gene copy number was assessed through the use of TaqMan probe quantitative PCR–based protocols [12]. All primers were synthesized by Thermo Electron.

RFLP analysis products were separated by electrophoresis in 2% agarose gels with ethidium bromide and were visualized under UV transillumination (GelDoc System; Bio-Rad).

Statistical analysis

Data from patient samples were analyzed using GraphPad QuickCalcs software (GraphPad Software). Proportions were compared using Fisher's exact test for the association of treatment type with SNPs. The complete set of baseline data (D_0 for the AL arm + D_0 for the SP arm) was used as a common comparator group for both arms of the drug trial. Statistical significance was denoted by $P \leq .05$. When mixed infections were evaluated, all calculations were performed by comparing carriers of the selected allele (including pure allele carriers and mixed infections) with parasites carrying the alternative allele in pure form.

P. falciparum propagation in vitro

All parasite lines were grown at 37°C in human red blood cells at a hematocrit of 3%–4%, by use of RPMI 1640 medium supplemented with 0.5% Albumax, 0.25% sodium bicarbonate, and 0.01 mg/mL gentamicin. Cultures were maintained in an atmosphere of 90% N_2, 5% O_2, and 5% CO_2.

In vitro drug assays

Parasite susceptibilities to lumefantrine were measured in vitro using 72-h ^{3}H-hypoxanthine assays with serial drug dilutions, as described elsewhere [24]. IC_{50} and IC_{90} values were determined by linear extrapolation. For statistical analyses, 2-tailed unpaired Student's *t* tests, as well as nonparametric Mann-Whitney *U* tests, were performed.

RESULTS

In total, 38 of the 50 children who were treated with AL experienced recurrent infections during the 42-day follow-up. Of these 38 children, 37 had sufficient parasite material to be included in the analysis. Of the 56 children included in the SP treatment arm, 39 had recurrent infections [2].

In the AL arm, only 2 recrudescences were recorded, which precluded a meaningful analysis of recrudescence versus reinfection. Hence, all breakthrough infections in the AL arm were simply considered to be recurrent and were treated equally. As for the SP arm, 16 recrudescences were identified. No selection for either *pfcrt* or *pfmdr1* variants was observed when SP recrudescences were compared with reinfections. These infections were also grouped together as recurrent infections.

The frequency of parasites carrying the *pfcrt* K76 allele (in pure form or mixed with 76T) increased from a baseline prevalence of 48.0% to 86.5% ($P < .0001$) among all recurrent infections in persons in the AL arm (table 1 and figure 1) and to 85.7% ($P = .0001$) among only reinfections occurring in persons in the AL arm. A statistically significant selection was also observed for *pfmdr1* N86, which displayed an increase in frequency from 55.9% at baseline to 75.7% among all recurrent infections ($P = .048$) (table 1 and figure 1), whereas the frequency increased to 74.3% among only reinfections occurring in the AL arm ($P = .071$). The frequencies of the *pfmdr1* 184F and D1246 alleles remained unaltered (table 1 and figure 1). The SP arm revealed no changes in the frequency of *pfcrt* K76T or *pfmdr1* N86Y in recurrent infections (table 1). The *pfmdr1* 1034 and 1042 SNPs, as well as the *pfcrt* 163 SNPs, showed no variation in either treatment.

Our sequencing procedures also revealed a novel nonsynonymous *pfmdr1* W1031R SNP (**T**GG→**C**GG) located in a predicted transmembrane domain [25]. The new SNP, which was

observed in 2 separate PCR amplification and sequencing studies, was present in 1 infection at baseline but was not detected among the recurrent infections in the group given AL. Finally, an increased number of *pfmdr1* gene copies was not detected in any of the 89 infections noted at baseline or in the 29 recurrent infections noted in the AL treatment arm that were successfully analyzed in this Tanzanian cohort.

To directly test the contribution of mutant *pfcrt* to susceptibility to lumefantrine under controlled in vitro conditions, we assayed *P. falciparum* lines that had been genetically engineered to express distinct *pfcrt* alleles, rather than the endogenous *pfcrt* allele in isogenic background. One set was produced in the CQ-susceptible GC03 line—a progeny of the HB3 × Dd2 genetic cross [26]. This set enabled us to compare clones expressing the wild-type CQ-susceptible allele ($C2^{GC03}$), the CQ-resistant Dd2 allele typical of Asian and African CQ-resistant strains ($C4^{Dd2}$), and the CQ-resistant 7G8 allele observed in South America, the Oceanic region, and India ($C6^{7G8}$) [19]. The Dd2 and 7G8 alleles contain 8- and 5-point mutations (M74I/N75E/K76T/A220S/Q271E/N326S/I356T/R371I and C72S/K76T/A220S/N326D/I356L), respectively, compared with the wild-type allele. To test the observed clinical association between the K76T mutation and susceptibility to lumefantrine, we also assayed clones generated in the Dd2 and 7G8 lines, which expressed either the parental allele ($C\text{-}1^{Dd2}$ and $C\text{-}1^{7G8}$) or an allele in which the mutant 76T codon had been replaced with the wild-type K76 codon (known as the $T76K\text{-}1^{Dd2}$ and $T76K\text{-}1^{7G8}$ “back mutant” clones, respectively). Recombinant clones were assayed in vitro on 5–8 separate occasions, and parental controls (GC03, Dd2, and 7G8) were assayed on 7–12 separate occasions.

These in vitro assays revealed that CQ-resistant lines with mutant *pfcrt* alleles carrying the K76T mutation were uniformly more susceptible to lumefantrine (table 2). $C4^{Dd2}$ and $C6^{7G8}$, expressing the 2 most prevalent alleles of mutant *pfcrt* worldwide, both showed a ~35% decrease in lumefantrine IC_{50} values, compared with $C2^{GC03}$ ($P < .05$ and $P < .01$, respectively, by the Mann-Whitney *U* test). The results achieved using the back-mutant clones demonstrated that the K76T mutation itself is a large mediator of this increased susceptibility. Both the $T76K\text{-}1^{Dd2}$ and $T76K\text{-}1^{7G8}$ clones were 2-fold less susceptible to lumefantrine than were their recombinant counterparts (for both, $P < .01$, by the Mann-Whitney *U* test). In the parental lines, we also observed substantially lower lumefantrine IC_{50} values in Dd2 and 7G8, compared with GC03.

DISCUSSION

Coartem, a fixed formulation of artemether and lumefantrine, is currently the most successful and widely implemented ACT and recently has been adopted as first- or second-line chemotherapy for the treatment of uncomplicated malaria by a large number of national malaria control programs in sub-Saharan Africa [27]. This combination, referred to as “AL” in the present study, has consistently demonstrated high efficacy. Nevertheless, the high levels of parasite transmission in most parts of sub-Saharan Africa dictate that reinfections will often occur after cessation of AL treatment [1–3]. This creates a risk for selection of reinfecting lumefantrine-tolerant/-resistant parasites after treatment, when the slowly eliminated lumefantrine remains at subtherapeutic concentrations. Previous data from some of the investigators involved in the present study [11]—data subsequently confirmed by others [13, 14]—have shown a consistent pattern of detectable increases in the frequency of drug resistance–associated *pfmdr1* haplotypes among recurrent infections after AL treatment. Because these recurrent parasites represent reinfections in all but a few cases, the data have been interpreted as a lumefantrine-driven selection for variant *pfmdr1* alleles.

In the present study, we report significant selection of the *pfcrt* K76 allele after AL treatment, which, to our knowledge, represents the first report of a significant in vivo association of this

gene with an ACT containing an arylaminoalcohol (a group of drugs that includes lumefantrine, MQ, and HF). This selection was not observed in our preceding studies, probably because of the rarity of the K76 allele in Zanzibar [1,11,12], as opposed to the present study site on mainland Tanzania, where ~50% of the analyzed infections harbored K76 allele carriers. As discussed elsewhere [12], the high efficacy of AL precludes the collection of statistically meaningful numbers of recrudescent parasites for analysis. Hence, the conclusions of the present study are mainly considered to be applicable to parasites causing reinfection (i.e., formally "lumefantrine-tolerant" parasites, as opposed to "lumefantrine-resistant" parasites [15]).

The selection for *pfcrt* K76 in the recurrent infections observed in the present study is consistent with earlier in vitro data supporting a role for this gene in *P. falciparum* susceptibility to arylaminoalcohols. After allele exchange at the *pfcrt* amino acid 76 position, isogenic clones have been shown to acquire different susceptibilities to MQ and HF. In the first of these experiments, exchanging the K76 for 76T (in the CQ-susceptible GC03 background) increased parasite in vitro susceptibility to MQ by 2- to 3-fold [19]. In a set of reverse experiments, the replacement of 76T with K76 decreased the susceptibility of the parasites to MQ, albeit to a lesser extent (1.5- to 2-fold) [20] (table 2). In another study, *P. falciparum* lines selected in vitro for HF resistance were shown to have acquired *pfcrt* mutations that included S163R, which was also associated with MQ resistance [22]. Finally, in vitro development of resistance to CQ in the Sudanese 106/1 clone (which already harbored 7 of the 8 Dd2 *pfcrt* mutations, lacking only K76T) resulted in novel *pfcrt* SNPs at position 76 (K76I/N) [17,21]. These novel K76I/N SNPs were shown to significantly increase susceptibility to MQ and HF [21].

Our present research extends these observations by documenting a 2-fold increase in susceptibility to lumefantrine in parasites expressing the K76T mutation (compare C-1^{Dd2} with T76K-1^{Dd2} and compare C-1^{7G8} with T76K-1^{7G8}). Interestingly, 7G8 was 2-fold more susceptible than Dd2, a finding that, on the basis of our data, cannot be attributed to differences between these *pfcrt* alleles. These lines also differ in their *pfmdr1* point mutations (with Dd2 having the 86Y/Y184/S1034/N1042/D1246 haplotype and with 7G8 having the N86/184F/1034C/1042D/1246Y haplotype) as well as their copy number (3–4 copies in our Dd2 line, compared with 1 copy in 7G8). Both types of genetic changes in *pfmdr1* are known to affect susceptibility to lumefantrine. Other, as yet undefined mediators may also differ between 7G8 and Dd2. Overall, our data clearly define the *pfcrt* K76T mutation as a major determinant that contributes to decreased susceptibility to lumefantrine on distinct genetic backgrounds.

Our results also show that the 2 globally most prevalent mutant *pfcrt* alleles, Dd2 and 7G8, both increase susceptibility to lumefantrine (compare C4^{Dd2} and C6^{7G8} with the isogenic CQ-susceptible C2^{GC03} line). In this regard, lumefantrine is an ideal ACT partner drug in areas of high resistance to CQ. Interestingly, mutant *pfcrt* alleles have also previously been found to significantly increase susceptibility to artemisinin [19]. This finding indicates an important dual benefit of using AL in areas with a high prevalence of mutant *pfcrt* conferring resistance to CQ.

Mutant PfCRT is thought to efflux compounds out of the DV into the cytoplasm. For CQ, which interferes with the detoxification of heme moieties inside the DV, resistance is thought to be achieved by mutant PfCRT-mediated export of drug away from its hematin target [25]. CQ resistance has been shown to critically depend on the *pfcrt* K76T mutation [20]. The observation that parasites carrying the *pfcrt* K76T mutation are more susceptible to lumefantrine and MQ suggests that those compounds might also be transported by mutant PfCRT out of the DV and, consequently, might exert their action in the parasite cytoplasm.

For *pfmdr1* and its encoded ABC-transporter Pgh-1, transport studies have shown that the *pfmdr1* copy number is associated with Fluo-4 accumulation inside the DV [28]. Moreover, it has been shown that the *pfmdr1* gene expressed in *Saccharomyces cerevisiae* functionally complements an exporter in the plasma membrane [29], with the nucleotide-binding domains on the cytosolic side [30]. In the DV membrane, Pgh-1 is situated with the nucleotide-binding domains in the cytoplasm [31,32], indicating a transport direction from the cytoplasm into the DV. Thus, Pgh-1 may function as a drug importer. For drugs such as CQ that have their target inside the DV, an increased number of *pfmdr1* copies might therefore be expected to result in increased drug susceptibility. In line with this hypothesis, selection of high-level CQ resistance has been observed to result in *pfmdr1* deamplification [33]. For drugs that have their target outside the DV, increased numbers of *pfmdr1* copies might therefore result in decreased susceptibility. The observed association between an increased *pfmdr1* copy number and treatment failures after AL [8], MQ, or artesunate-MQ therapy [9] is therefore another indication that the target of lumefantrine and MQ might lie outside the DV.

The CQ susceptibility markers *pfcrt* K76 and *pfmdr1* N86 [18] and increases in *pfmdr1* copy number have all been associated with a decreased susceptibility to both lumefantrine and MQ [7–9,11,19–21,34,35]. Thus, substantial evidence points to cross-resistance between the 2 arylaminoalcohols. In Southeast Asia, decreased efficacy of artesunate-MQ treatment has been reported [36], presumably because of MQ resistance caused by an increased *pfmdr1* copy number [37]. Although an increased *pfmdr1* copy number seems to be rare in Africa, it has previously been detected in Gabon in a subset of patients treated with a low dose of MQ. This suggests a risk for selection and spread of multicopy *pfmdr1* in Africa after the ongoing wide-scale deployment of AL [10]. This finding, combined with our observation of selection of *pfcrt* K76 and *pfmdr1* N86 alleles, suggests that AL tolerance or resistance could emerge in Africa. This scenario would have major public health implications for AL as a malaria treatment, in terms of its ability to effectively and sustainably reduce disease-associated morbidity and mortality.

In the present study, we compared selection of *pfmdr1* and *pfcrt* mutations that occurred in association with AL treatment with selection that occurred in association with treatment with SP, a drug for which resistance is established to be unrelated to genetic alterations in these genes. Because no selection was observed for the analyzed SNPs in the SP arm, the present study supports the argument that *pfmdr1* N86 and, now, *pfcrt* K76 are specifically associated with a decreased susceptibility to lumefantrine, allowing a more successful reinfection of the human host in the period corresponding to slowly decreasing lumefantrine concentrations [11,15].

The lack of *pfcrt* K76 selection in the SP treatment arm is in apparent contrast to the documented replacement of the 76T allele by K76 in regions where SP has been introduced to replace CQ, as has been illustrated by reports from Malawi [38,39]. In this regard, we speculate that harboring *pfcrt* K76 presents a relatively small fitness advantage, compared with harboring *pfcrt* 76T. In the absence of CQ pressure over several years, the progressive replacement becomes detectable in the overall population, ultimately overtaking the parasite population harboring the K76T mutation [38–41].

In conclusion, we identified the *pfcrt* K76 allele as a further potential marker of decreased susceptibility to lumefantrine in vivo, in addition to the established *pfmdr1* SNPs [10–12]. Importantly, *pfcrt* encoding the K76T mutation, which confers CQ resistance in vitro and is a highly sensitive marker of CQ treatment failure in vivo, also increases in vitro susceptibility to artemisinin [19]. These findings highlight a substantial benefit of using AL in areas with a high prevalence of CQ-resistant *P. falciparum* malaria. Defining the interrelationships between drug-determinant polymorphisms and changes in susceptibility to the various antimalarials

will be important in ongoing efforts to best manage the implementation of different ACTs and reduce the dissemination and influence of drug-resistant malaria.

Acknowledgments

Financial support: Swedish Development Cooperation Agency–Department for Research Cooperation (grants SWE 2005–017 and SWE 2005–004596); Research and Development Unit, Sörmland Count Council, Sweden (project grant 82090); Goljes Foundation (project grants 248/04 and 317/05); Fundacão para a Ciência e Tecnologia, Portugal (PhD grant SFRH/BD/28393/2006 to M.I.V.); US National Institutes of Health (grant R01 AI50234 to D.A.F.).

References

1. Martensson A, Strömberg J, Sisowath C, et al. Efficacy of artesunate plus amodiaquine versus that of artemether-lumefantrine for the treatment of uncomplicated childhood *Plasmodium falciparum* malaria in Zanzibar, Tanzania. Clin Infect Dis 2005;41:1079–1086. [PubMed: 16163624]
2. Martensson A, Ngasala B, Ursing J, et al. Influence of consecutive-day blood sampling on polymerase chain reaction–adjusted parasitological cure rates in an antimalarial-drug trial conducted in Tanzania. J Infect Dis 2007;195:597–601. [PubMed: 17230421]
3. Zongo I, Dorsey G, Rouamba N, et al. Randomized comparison of amodiaquine plus sulfadoxine-pyrimethamine, artemether-lumefantrine, and dihydroartemisinin-piperaquine for the treatment of uncomplicated *Plasmodium falciparum* malaria in Burkina Faso. Clin Infect Dis 2007;45:1453–1461. [PubMed: 17990228]
4. Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF. Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. Nature 2000;403:906–909. [PubMed: 10706290]
5. Sidhu AB, Valderramos SG, Fidock DA. *pfmdr1* mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in *Plasmodium falciparum*. Mol Microbiol 2005;57:913–926. [PubMed: 16091034]
6. Anderson TJ, Nair S, Qin H, et al. Are transporter genes other than the chloroquine resistance locus *(pfcrt)* and multidrug resistance gene *(pfmdr)* associated with antimalarial drug resistance? Antimicrob Agents Chemother 2005;49:2180–2188. [PubMed: 15917511]
7. Duraisingh MT, Jones P, Sambou I, von Seidlein L, Pinder M, Warhurst DC. The tyrosine-86 allele of the *pfmdr1* gene of *Plasmodium falciparum* is associated with increased sensitivity to the anti-malarials mefloquine and artemisinin. Mol Biochem Parasitol 2000;108:13–23. [PubMed: 10802315]
8. Price RN, Uhlemann AC, van Vugt M, et al. Molecular and pharmacological determinants of the therapeutic response to artemether-lumefantrine in multidrug-resistant *Plasmodium falciparum* malaria. Clin Infect Dis 2006;42:1570–1577. [PubMed: 16652314]
9. Price RN, Uhlemann AC, Brockman A, et al. Mefloquine resistance in *Plasmodium falciparum* and increased *pfmdr1* gene copy number. Lancet 2004;364:438–447. [PubMed: 15288742]
10. Uhlemann AC, Ramharter M, Lell B, Kremsner PG, Krishna S. Amplification of *Plasmodium falciparum* multidrug resistance gene 1 in isolates from Gabon. J Infect Dis 2005;192:1830–1835. [PubMed: 16235185]
11. Sisowath C, Strömberg J, Mårtensson A, et al. In vivo selection of *Plasmodium falciparum pfmdr1* 86N coding alleles by artemether-lumefantrine (Coartem). J Infect Dis 2005;191:1014–1017. [PubMed: 15717281]
12. Sisowath C, Ferreira PE, Bustamante LY, et al. The role of *pfmdr1* in *Plasmodium falciparum* tolerance to artemether-lumefantrine in Africa. Trop Med Int Health 2007;12:736–742. [PubMed: 17550470]
13. Dokomajilar C, Nsobya SL, Greenhouse B, Rosenthal PJ, Dorsey G. Selection of *Plasmodium falciparum pfmdr1* alleles following therapy with artemether-lumefantrine in an area of Uganda where malaria is highly endemic. Antimicrob Agents Chemother 2006;50:1893–1895. [PubMed: 16641472]
14. Humphreys GS, Merinopoulos I, Ahmed J, et al. Amodiaquine and artemether-lumefantrine select distinct alleles of the *Plasmodium falciparum mdr1* gene in Tanzanian children treated for uncomplicated malaria. Antimicrob Agents Chemother 2007;51:991–997. [PubMed: 17194834]

15. Hastings IM, Ward SA. Coartem (artemether-lumefantrine) in Africa: the beginning of the end? J Infect Dis 2005;192:1303–1304. [PubMed: 16136476]author reply, 1304–5
16. Hayward R, Saliba KJ, Kirk K. *pfmdr1* mutations associated with chloroquine resistance incur a fitness cost in *Plasmodium falciparum*. Mol Microbiol 2005;55:1285–1295. [PubMed: 15686571]
17. Fidock DA, Nomura T, Talley AK, et al. Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. Mol Cell 2000;6:861–871. [PubMed: 11090624]
18. Djimdé A, Doumbo OK, Cortese JF, et al. A molecular marker for chloroquine-resistant falciparum malaria. N Engl J Med 2001;344:257–263. [PubMed: 11172152]
19. Sidhu AB, Verdier-Pinard D, Fidock DA. Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by *pfcrt* mutations. Science 2002;298:210–213. [PubMed: 12364805]
20. Lakshmanan V, Bray PG, Verdier-Pinard D, et al. A critical role for PfCRT K76T in *Plasmodium falciparum* verapamil-reversible chloroquine resistance. EMBO J 2005;24:2294–2305. [PubMed: 15944738]
21. Cooper RA, Ferdig MT, Su XZ, et al. Alternative mutations at position 76 of the vacuolar transmembrane protein PfCRT are associated with chloroquine resistance and unique stereospecific quinine and quinidine responses in *Plasmodium falciparum*. Mol Pharmacol 2002;61:35–42. [PubMed: 11752204]
22. Johnson DJ, Fidock DA, Mungthin M, et al. Evidence for a central role for PfCRT in conferring *Plasmodium falciparum* resistance to diverse antimalarial agents. Mol Cell 2004;15:867–877. [PubMed: 15383277]
23. Veiga MI, Ferreira PE, Björkman A, Gil JP. Multiplex PCR-RFLP methods for *pfcrt*, *pfmdr1* and *pfdhfr* mutations in *Plasmodium falciparum*. Mol Cell Probes 2006;20:100–104. [PubMed: 16460912]
24. Fidock DA, Nomura T, Wellems TE. Cycloguanil and its parent compound proguanil demonstrate distinct activities against *Plasmodium falciparum* malaria parasites transformed with human dihydrofolate reductase. Mol Pharmacol 1998;54:1140–1147. [PubMed: 9855645]
25. Valderramos SG, Fidock DA. Transporters involved in resistance to antimalarial drugs. Trends Pharmacol Sci 2006;27:594–601. [PubMed: 16996622]
26. Wellems TE, Panton LJ, Gluzman IY, et al. Chloroquine resistance not linked to *mdr*-like genes in a *Plasmodium falciparum* cross. Nature 1990;345:253–255. [PubMed: 1970614]
27. World Health Organization. Treatment policies. [Accessed 20 May 2008]. Available at http://www.who.int/malaria/treatmentpolicies.html
28. Rohrbach P, Sanchez CP, Hayton K, et al. Genetic linkage of *pfmdr1* with food vacuolar solute import in *Plasmodium falciparum*. EMBO J 2006;25:3000–3011. [PubMed: 16794577]
29. Volkman SK, Cowman AF, Wirth DF. Functional complementation of the *ste6* gene of *Saccharomyces cerevisiae* with the *pfmdr1* gene of *Plasmodium falciparum*. Proc Natl Acad Sci USA 1995;92:8921–8925. [PubMed: 7568044]
30. Michaelis S. STE6, the yeast α-factor transporter. Semin Cell Biol 1993;4:17–27. [PubMed: 8095825]
31. Duraisingh MT, Cowman AF. Contribution of the *pfmdr1* gene to anti-malarial drug-resistance. Acta Trop 2005;94:181–190. [PubMed: 15876420]
32. Karcz SR, Galatis D, Cowman AF. Nucleotide binding properties of a P-glycoprotein homologue from *Plasmodium falciparum*. Mol Biochem Parasitol 1993;58:269–276. [PubMed: 8097560]
33. Barnes DA, Foote SJ, Galatis D, Kemp DJ, Cowman AF. Selection for high-level chloroquine resistance results in deamplification of the *pfmdr1* gene and increased sensitivity to mefloquine in *Plasmodium falciparum*. EMBO J 1992;11:3067–3075. [PubMed: 1353446]
34. Price RN, Cassar C, Brockman A, et al. The *pfmdr1* gene is associated with a multidrug-resistant phenotype in *Plasmodium falciparum* from the western border of Thailand. Antimicrob Agents Chemother 1999;43:2943–2949. [PubMed: 10582887]
35. Pickard AL, Wongsrichanalai C, Purfield A, et al. Resistance to antimalarials in Southeast Asia and genetic polymorphisms in *pfmdr1*. Antimicrob Agents Chemother 2003;47:2418–2423. [PubMed: 12878499]
36. White NJ. Qinghaosu (artemisinin): the price of success. Science 2008;320:330–334. [PubMed: 18420924]

37. Wongsrichanalai C, Meshnick SR. Declining artesunate-mefloquine efficacy against falciparum malaria on the Cambodia-Thailand border. Emerg Infect Dis 2008;14:716–769. [PubMed: 18439351]

38. Kublin JG, Cortese JF, Njunju EM, et al. Reemergence of chloroquine-sensitive *Plasmodium falciparum* malaria after cessation of chloroquine use in Malawi. J Infect Dis 2003;187:1870–1875. [PubMed: 12792863]

39. Mita T, Kaneko A, Lum JK, et al. Recovery of chloroquine sensitivity and low prevalence of the *Plasmodium falciparum* chloroquine resistance transporter gene mutation K76T following the discontinuance of chloroquine use in Malawi. Am J Trop Med Hyg 2003;68:413–415. [PubMed: 12875289]

40. Mita T, Kaneko A, Lum JK, et al. Expansion of wild type allele rather than back mutation in *pfcrt* explains the recent recovery of chloroquine sensitivity of *Plasmodium falciparum* in Malawi. Mol Biochem Parasitol 2004;135:159–163. [PubMed: 15287598]

41. Laufer MK, Thesing PC, Eddington ND, et al. Return of chloroquine antimalarial efficacy in Malawi. N Engl J Med 2006;355:1959–1966. [PubMed: 17093247]

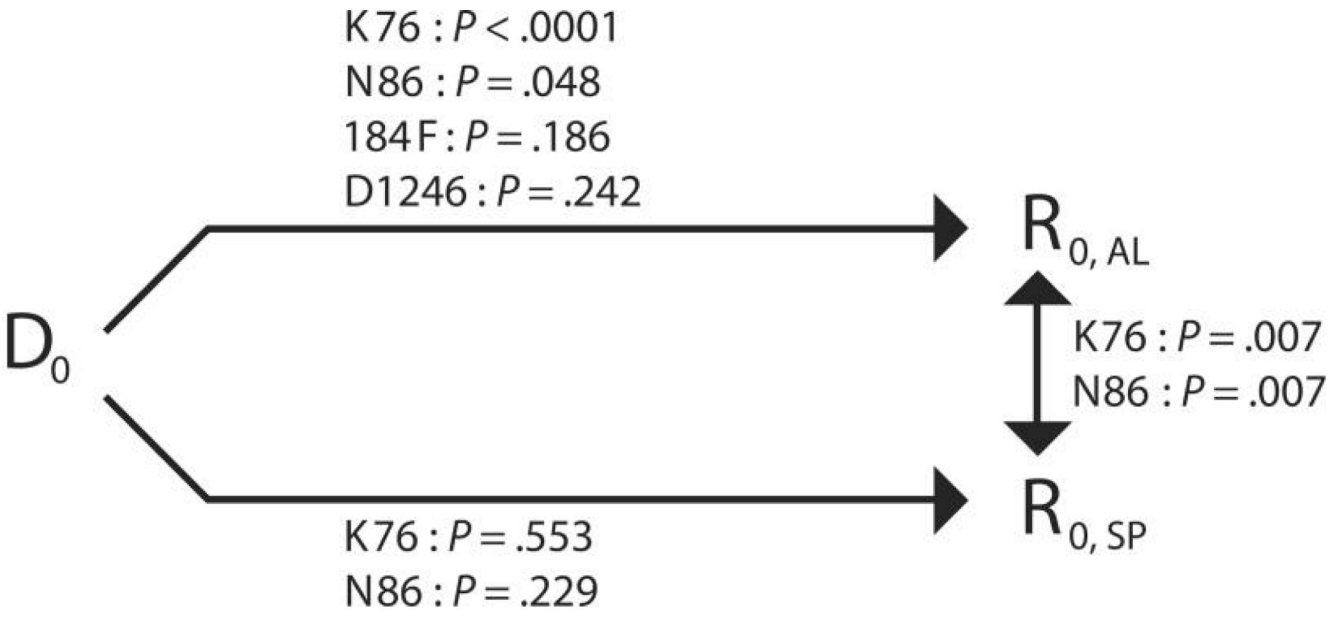

Figure 1.
Set of comparisons (conducted using Fisher's exact test [see Subjects, Materials, and Methods]) between infections at baseline (D_0) and all recurrent infections (R_0) concerning the chloroquine resistance transporter gene *(pfcrt)* K76 allele and the multidrug resistance gene 1 *(pfmdr1)* N86, 184F, and D1246 alleles. The *pfcrt* S163 residue, as well as the *pfmdr1* S1034 and N1042 residues, had a prevalence of 100% in this setting, as did single-copy *pfmdr1*. AL, artemether-lumefantrine; SP, sulfadoxine-pyrimethamine.

Table 1

Frequencies of the analyzed single-nucleotide polymorphisms (SNPs), both before the administration of the drug (D_0) and in recurrent infections (R_0).

	Frequency, (pure + mix)/all [95% CI][a]		
		R_0	
SNP	D_0	AL	SP
pfcrt K76	(26 + 23)/102 = 0.480 [0.386–0.576]	(22 + 10)/37 = 0.865 [0.716–0.946]	(12 + 7)/34 = 0.559 [0.394–0.711]
pfmdr1 N86	(27 + 30)/102 = 0.559 [0.462–0.651]	(21 + 7)/37 = 0.757 [0.597–0.868]	(8 + 6)/33 = 0.424 [0.272–0.592]
pfmdr1 184F	(0 + 25)/100 = 0.250 [0.175–0.344]	(3 + 10)/34 = 0.382 [0.239–0.550]	NA
pfmdr1 D1246	(50 + 24)/98 = 0.755 [0.661–0.830]	(21 + 9)/35 = 0.857 [0.702–0.942]	NA

NOTE. The D_0 data for both treatment arms were pooled at baseline, because no significant differences were found between the 2 groups for any of the analyzed SNPs. AL, artemether-lumefantrine; CI, confidence interval; NA, not analyzed; SP, sulfadoxine-pyrimethamine.

[a] "All" denotes the total no. of successful analyses. The 95% CI was determined using the modified Wald method (GraphPad Software; GraphPad).

Table 2

Lumefantrine susceptibility levels in chloroquine resistance transporter gene *(pfcrt)*–modified and control lines.

LMF IC_{50}, nmol/L[a]	Recombinant	Parental line	PfCRT haplotype									CQ[b]		ARTM IC_{50},[b] nmol/L	MQ IC_{50},[b] nmol/L
			72	74	75	76	220	271	326	356	371	IC_{50}, nmol/L	Phenotype		
85.5 ± 7.2	No	...	C	M	N	K	A	Q	N	I	R	29.2 ± 2.0	Susc.	39.4 ± 6.9	52.6 ± 5.4
55.2 ± 9.6	No	...	C	I	E	T	S	E	S	T	I	195.1 ± 25.3	Res.	46.3 ± 7.6	46.8 ± 2.0
27.0 ± 4.1	No	...	S	M	N	T	S	Q	D	L	R	140.4 ± 15.0	Res.	21.6 ± 6.3	9.6 ± 1.3
74.0 ± 7.7	Yes	GC03	C	M	N	K	A	Q	N	I	R	22.9 ± 2.8	Susc.	39.1 ± 7.0	54.7 ± 1.5
48.7 ± 4.3[c]	Yes	GC03	C	I	E	T	S	E	S	T	I	147.4 ± 13.3[d]	Res.	12.6 ± 1.8[d]	33.1 ± 3.1[d]
46.3 ± 4.6[d]	Yes	GC03	S	M	N	T	S	Q	D	L	R	126.9 ± 17.2[d]	Res.	17.2 ± 3.0[d]	27.3 ± 3.4[d]
73.2 ± 10.5[d]	Yes	Dd2	C	I	E	K	S	E	S	T	I	22.1 ± 2.1[d]	Susc.	40.4 ± 11.7	27.2 ± 8.0[c]
33.7 ± 5.0	Yes	Dd2	C	I	E	T	S	E	S	T	I	160.7 ± 28.9	Res.	32.9 ± 6.6	36.9 ± 4.2
68.3 ± 11.7[d]	Yes	7G8	C	I	E	K	S	Q	D	L	R	23.9 ± 4.6[e]	Susc.	13.8 ± 3.2	3.6 ± 0.7
33.6 ± 5.5	Yes	7G8	S	M	N	T	S	Q	D	L	R	108.8 ± 15.9	Res.	7.4 ± 2.1	3.1 ± 0.9

RTM, artemisinin; CQ, chloroquine; LMF, lumefantrine; MQ, mefloquine; Res., resistant; Susc., susceptible.

50 values are expressed as the mean value (±SE) for *pfcrt*–modified and control lines. Data were derived from 5–12 independent assays performed in duplicate. (Results from Mann-Whitney ompared mutants and recombinant controls).

TM, and MQ IC_{50} values (from at least 3 independent assays performed in duplicate) are expressed as the mean value (±SE) and are reproduced from [19] for the top 6 lines and from [20] for n 4 lines.

[e] $P < .001$.