Evidence for linkage of pfmdr1, pfcrt, and pfk13 polymorphisms to lumefantrine and mefloquine susceptibilities in a Plasmodium falciparum cross

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ARTICLE INFO

Keywords:
Malaria
Artemisinin-based combination chemotherapy
Drug resistance
Halofantrine
Quantitative trait loci analysis

ABSTRACT

Background: Lumefantrine and mefloquine are used worldwide in artemisinin-based combination therapy (ACT) of malaria. Better understanding of drug susceptibility and resistance is needed and can be obtained from studies of genetic crosses.

Methods: Drug response phenotypes of a cross between Plasmodium falciparum lines 803 (Cambodia) and GB4 (Ghana) were obtained as half-maximal effective concentrations (EC 50 s) and days to recovery (DTR) after 24 h exposure to 500 nM lumefantrine. EC 50 of mefloquine, halofantrine, chloroquine, and dihydroartemisinin were also determined. Quantitative trait loci (QTL) analysis and statistical tests with candidate genes were used to identify polymorphisms associated with response phenotypes.

Results: Lumefantrine EC 50 s averaged 5.8-fold higher for the 803 than GB4 parent, and DTR results were 3–5 and 16–18 days, respectively. In 803 × GB4 progeny, outcomes of these two lumefantrine assays showed strong inverse correlation; these phenotypes also correlated strongly with mefloquine and halofantrine EC 50 s. By QTL analysis, lumefantrine and mefloquine phenotypes mapped to a chromosome 5 region containing codon polymorphisms N86Y and Y184F in the P. falciparum multidrug resistance 1 protein (PfMDR1). Statistical tests of candidate genes identified correlations between inheritance of PfK13 Kelch protein polymorphism C580Y (and possibly K189T) and lumefantrine and mefloquine susceptibilities. Correlations were detected between lumefantrine and chloroquine EC 50 s and polymorphisms N326S and I356T in the CVIET-type P. falciparum chloroquine resistance transporter (PfCRT) common to 803 and GB4.

Conclusions: Correlations in this study suggest common mechanisms of action in lumefantrine, mefloquine, and halofantrine responses. PfK13 as well as PfMDR1 and PfCRT polymorphisms may affect access and/or action of these arylaminoalcohol drugs at locations of hemoglobin digestion and heme metabolism. In endemic regions, pressure from use of lumefantrine or mefloquine in ACTs may drive selection of PfK13 polymorphisms along with versions of PfMDR1 and PfCRT associated with lower susceptibility to these drugs.

1. Introduction

The availability of effective, safe, and affordable antimalarial drugs is increasingly threatened by the emergence and spread of drug resistant parasites (Phillips et al., 2017). Resistance to partner drugs in artemisinin-based combination therapies (ACTs) is an important case in point, as ACTs are presently recommended worldwide as first-line medicines for the treatment of malaria (World Health Organization, 2015). Artemether-lumefantrine (AL, Coartem®) was the first ACT produced under international good manufacturing practices. Since its approval by the United States Food and Drug Administration in 2009, AL has also become available as Coartem® Dispersible for pediatric use and treatment.
is now the most widely used ACT for the treatment of uncomplicated
*Plasmodium falciparum* malaria, particularly in Africa (Premji, 2009;
Nzila et al., 2012). Efficacies of AL treatment in large studies have
generally been better than 95% (Makanga and Krudsood, 2009; Premji,
2009), although recrudescences are reported in nonimmune adults
and travelers despite appropriate lumefantrine (LUM) concentrations
in the blood (Farnert et al., 2012; Sonden et al., 2017). Because LUM is a
lipophilic arylmethylaolcohol that has limited absorption on an empty
stomach, AL is recommended to be taken with a fatty food meal to
achieve adequate bioavailability (White et al., 1999; Denis et al., 2006;
Premji, 2009). Additionally, the importance of adherence to the treat-
ment regimen has been quantitatively demonstrated in outcome
modeling studies (Challenger et al., 2017).

While much about the action of LUM as an antimalarial has yet to be
elucidated, evidence suggests the drug can inhibit hemozoin formation
in the digestive vacuole (DV) of the erythrocystic-stage malaria parasite,
where the hemozoin serves as a crystalline repository for the seques-
tration and detoxification of ferri-protoporphyrin heme molecules
released by hemoglobin digestion (Pradines et al., 1999; Combrinck
et al., 2013). LUM response variations result from *P. falciparum* coding
polymorphisms and copy number differences in the *P. falciparum*
multidrug resistance 1 gene (*pfmdr1*) including: (1) codon N86Y, Y184F,
and D1246Y substitutions relative to the 3D7 ‘wild-type’ sequence
(Duraisingh et al., 2000; Sisowath et al., 2005; Venkatesan et al., 2014;
Wurz et al., 2014; Baraka et al., 2015); and (2) amplifications of regions
of *P. falciparum* chromosome 5 containing the *pfmdr1* gene (Price et al.,
2006; Sidhu et al., 2006; Nair et al., 2007; Gadalla et al., 2011; Ven-
katesan et al., 2014). Selection of the *pfmdr1* N86/184F/D1246 haplo-
type has been reported from studies of AL-treatments and
recrudescences in patients in Asia and Africa (Sisowath et al., 2005;
Venkatesan et al., 2014; Conrad et al., 2014). Likewise, selection of the wild-type K76
codon in the *P. falciparum* chloroquine resistance transporter gene, *pfcrt,*
has been associated with pressure from AL treatment in a number of
studies (Sisowath et al., 2009; Eyase et al., 2013; Conrad et al., 2014;
Venkatesan et al., 2014; Baraka et al., 2015). Dramatic increases in the
prevalence of wild-type *PfCRT* K76 and *PfMDR1* N86 were associated
with discontinuation of chloroquine (CQ) and deployment of AL in
western Kenya, although AL continued to be efficacious with these
changes (Achieng et al., 2015). EC_{50} of CQ and amodiaquine (AQ) have
been found to decrease reciprocally with increases in the EC_{50} of LUM
and mefloquine (MEF), and these reciprocal changes have been associ-
ated with opposite shifts in the prevalence of mutant vs. wild-type PICRT
and PfMDR1 polymorphisms (Humphreys et al., 2007; Eyase et al.,
2013; Venkatesan et al., 2014; Sondo et al., 2016).

Experimental crosses of *Plasmodium* parasites are a powerful means
to identify genes of drug resistance and other important malaria pheno-
types. With the human malaria parasites, crosses have enabled the
mapping and characterization of genes that determine in vivo responses of *P. falciparum* to antifolate antimalarials (Peterson et al., 1988, 1990;
Wang et al., 1997); the 4-aminoquinolines CQ and AQ along with their
active metabolites (Wellens et al., 1991; Fidoek et al., 2006; Så et al.,
2009); the arylmethylaolcohol drugs LUM, MEF, and halofantrine (HLF),
as well as the endoperoxide drugs artemisinin, arteleine, artether,
dihydroartemisinin, and artesunate (Duraisingh et al., 2000). Genetic
crosses have also supported the evaluations of in vivo phenotypes and
genetic determinants of *P. falciparum* artemisinin response and of *Plas-
modium* chloroquine (CQ) resistance in monkey models (Så et al., 2018, 2019).

Other biological investigations with *P. falciparum* crosses have led to the
discoveries of a major parasite ligand for species-specific erythrocyte
invasion (Hayton et al., 2008), family members of the channel that de-
termines nutrient uptake by parasitized erythrocytes (Nguiragool et al.,
2011), and a key molecule mediating parasite evasion of the mosquito
immune system (Molina-Cruz et al., 2013).

The present study was undertaken to assess LUM parasite suscepti-
bilities using two different drug response assays, compare these
susceptibilities with those of other arylmethylaolcohol drugs such as MEF
and HLF, and for search for genes that affect phenotypes of differential
susceptibility in a cross between clonal lines of Cambodian (803) and
Ghanaian (GB4) *P. falciparum* parasites (Så et al., 2018). The genetic
determinants of susceptibility in these two geographical regions are of
particular interest, as LUM and MEF phenotypes have been reported to
differ between Africa and Southeast Asia (Oduola et al., 1993; Dama
et al., 2017) and parasite susceptibilities to LUM and MEF as well as HLF
are known to correlate with one another (Basco et al., 1998; Pradines
et al., 2006; Basco and Ringwald, 2007; Eyase et al., 2013). Here we
describe findings consistent with previous reports of arylmethylaolcohol
drug responses mediated by PfMDR1 and PICRT polymorphisms, pro-
vide evidence that mutations in the *P. falciparum* Kelch 13 protein
(PfK13) associate with reduced parasite susceptibility to LUM and MEF,
and present a hypothesis that LUM and MEF may drive selection of
PfK13 580Y and perhaps other Kelch protein mutations in *P. falciparum*
populations pressured by ACTs containing these drugs.

2. Materials and methods

2.1. *P. falciparum* parasites of the 803 × GB4 cross and in vitro
cultivation

Production of the 803 × GB4 cross and recovery of the independent
recombinant progeny for genetic linkage studies was as previously
described (Så et al., 2018). Briefly, gametocytes were induced from
blood stage cultures in vitro, mixed, and fed to *Anopheles* mosquitoes.
After infection of the mosquitoes and confirmation of recombinant oo-
cysts, sporozoites were allowed to develop in the mosquito salivary
glands, purified, and cryopreserved. Sporozoite populations carrying
markers of both parents (sporozoites, like blood stages, are haploid)
were later thawed and inoculated into a *Pan troglodytes* chimpanzee
to produce broods of liver stage parasites and subsequent populations of
recombinant blood-stage progeny. Blood samples were collected 18–40
days after the inoculation and the parasites were cultivated briefly in
vitro before cloning by limiting dilution. After sorting and character-
ization of more than 400 parasite clones by microsatellite fingerprinting,
a subset of clones representing broods of independent recombinants was
identified and typed by microarray genome analysis with 3629 single
nucleotide polymorphisms.

Parasite lines from the 803 × GB4 cross were maintained in 10 mL
cultures with human O+ red blood cells (RBCs) (Virginia Blood Bank,
VA, USA) at ~5% hematocrit supplemented with complete media (CM)
composed of RPMI-1640 (Sigma-Aldrich; St. Louis, MO), 2.0% Albu-
MAX™ II wt/vol (Life Technologies™; Grand Island, NY), 0.02 mg/mL
gentamycin solution, and 0.21% sodium bicarbonate (KD Medical;
Columbia, MD) following established culture methods (Cramer et al.,
1997). Cultures were incubated at 37 °C in a 90% nitrogen/5% oxy-
gen/5% carbon dioxide environment. Parasitemia and stage distribu-
tions were monitored by thin blood smears methanol-fixed and stained
with 20% Giemsa for 15–30 min as well as by flow cytometry (Amar-
atunga et al., 2014). Synchronization of parasites was performed as
described (Lambros and Vanderberg, 1979) by suspension of the pellet
from 10 mL of cell culture (0.5 mL packed cells) for 5–10 min in 5 mL of
5% sorbitol wt/vol, followed by a 30 mL wash with CM.

2.2. 72-Hour half-maximal effective concentration (EC_{50}) assays

CQ, MEF, HLF, and LUM were purchased from Sigma-Aldrich (St.
Louis, MO). Stock solutions were stored at 5 mM or 10 mM in dime-
thylsulfoxide at −20 °C. The 72 h dose response assays followed previ-
ous methods using SYBR Green I and 96-well plates (Smilkstein et al.,
2004; Lane et al., 2018). Geometric mean half-maximal effective con-
centrations (EC_{50}) with confidence intervals were calculated from the
response curves using non-linear regression with variable slope on Prism
v.8 (GraphPad Software Inc.; La Jolla, CA). Assays were repeated for
each line a minimum of three times to obtain geometric mean and confidence interval.

2.3. Growth recovery times after a 24 h 500 nM LUM exposure

Parasites were cultivated to 2% parasitemia at 5% hematocrit with at least 70% ring stage parasites, then exposed for 24 h to 500 nM LUM (Sigma-Aldrich, St. Louis, MO). These parameters were chosen in view of the blood concentrations and frequency of parasite clearances reported 24 h after treatment (Valecha et al., 2012), thus approximating physiological conditions of exposure better in vitro than EC50 assays with LUM concentrations in the low nM range. Following this exposure, the cells were washed three times with 50 mL of CM and returned to culture with daily medium changes, maintaining 5% hematocrit until they again reached 2% parasitemia. Days to recovery (DTR) were counted as the number of days for parasites in culture to reach the parasitemia recorded at the beginning of each experiment. Assays for each parasite line were repeated a minimum of three times, with the exceptions of 36H9 and 40E7 (n = 2) to obtain mean and confidence interval.

2.4. Genome-wide quantitative trait-loci analysis and statistical evaluations

Quantitative trait-loci (QTL) analysis was performed using the R/qtl package as described (Broman et al., 2003). For the 803 × GB4 parents and for each of the studied progeny, the mean DTR and geometric mean EC50 measures were provided as phenotypes, and the corresponding genotypes of each parasite were entered from the previously established DNA microarray datasets of 3629 single-nucleotide polymorphisms (SNPs) across the genome (Sá et al., 2018). QTL searches for primary loci were performed using marker regression with 10 centimorgan (cM) spacing with these SNPs used for locus inheritance. P-values were determined from 1000 permutations. Significant (p < 0.05) logarithm of odds (LOD) scores were taken to indicate genetic linkage. LOD intervals were determined from the span of the peak exceeding the calculated significance cutoff.

Spearman’s rank correlations of EC50 and DTR phenotypes and Mann-Whitney U tests of candidate gene polymorphisms against these phenotypes were performed using Prism v.8.

2.5. Microsatellite genotyping of parasite lines

Genetic verification of parasite lines was performed by PCR amplification of 12 variable markers as described (Figan et al., 2018). Capillary electrophoresis was performed on an ABI 3730XL machine and analyzed by GeneMapper v4.3 software (Applied Biosystems; La Jolla, CA). Distinguishable sizes of each marker identify the 803 and GB4 parents. Each unique progeny clone (segregant) from 803 × GB4 cross is identified by its particular combination of these 12 markers.
3. Results and discussion

3.1. Drug response phenotypes of parents and progeny of the 803 x GB4 cross

LUM drug responses of the parental clones 803 and GB4 plus 22 of the independent recombinant progeny were assessed by standard EC_{50} assays. The geometric mean LUM EC_{50} of 803 was 5.8-fold greater than GB4 (3.21 nM, 95% Confidence Interval 2.80–3.66 nM vs. 0.55 nM, 95% CI 0.46–0.67 nM, respectively; Fig. 1A, Table S1). MEF, HLF, and CQ EC_{50}s were obtained along with those of LUM; dihydroartemisinin (DHA) responses were determined previously (Sá et al., 2018). Comparisons of the MEF and HLF responses showed that the Cambodian 803 line, as for LUM, was less susceptible than Ghanaian GB4 to these drugs: the geometric mean EC_{50} of 803 relative to GB4 were 2.9-fold greater with MEF and 4.6-fold greater with HLF, whereas these were 2.0-fold greater with CQ and 1.7-fold reduced with DHA (Table 1).

Geometric mean LUM EC_{50} of the individual 803 x GB4 parents and progeny were 0.55–5.21 nM (Table 1), a range that overlapped with those of 0.19–12.5 nM and 1.4–3.4 nM reported by Nsobya et al. (2010) and Dama et al. (2017) but was lower than the ranges from other studies by Basco et al. (1998) (95% CI 10.4–13.6 nM), Pradines et al. (2006) (5.7–8.2 nM), Mwai et al. (2009) (Interquartile Range 29–96 nM), Eyase et al. (2013) (IQR of 9.5–52.4 nM in 2011), and Wurtz et al. (2014) (95% CI 18.8–26.9 nM). Assay details such as timings of drug exposure, compositions of culture media, incubation conditions employed, and the status and synchrony of parasite populations are among potential reasons for the differences in these ranges. We note that the higher range EC_{50} values remain well below bloodstream levels achieved after a standard dose of LUM, although exposure times of 72 h approach the 3 day half-life of the drug (White et al., 1999; Valecha et al., 2012).

In the absence of accepted standards for LUM-resistant P. falciparum parasites, much remains to be resolved about the LUM levels indicative of drug sensitivity, tolerance, and resistance in these susceptibility assays. Reference parasites with benchmark phenotypes of LUM resistance have yet to be established either as culture-adapted lines from clinical treatment failures or as selected lines after LUM pressure in vitro.

To study the LUM responses of parasites after exposures closer to those of drug treatment in vitro (Valecha et al., 2012), we developed an assay that employs 500 nM LUM exposure (~100x EC_{50}) for 24 h. Outcomes of this assay were assessed by measuring the days to recovery (DTR) to starting parasitemia. Three to five days were required for the drug-exposed 803 parasites to recover in these assays, whereas the drug-exposed GB4 parasites required 16–18 days (Fig. 1B, Table S2).

Interestingly, no dormant forms were observed after exposure of the parasites to 500 nM LUM for 24 h, consistent with the report of Chavchich et al. (2016); further, in separate assessments, we were unable to find evidence for any reduced LUM susceptibility to the drug by the parasites that repopulated the culture after surviving 500 nM LUM exposure.

Twenty-two recombinant progeny from the 803 x GB4 cross were assessed by the LUM EC_{50} and DTR assays. In both of these assays, the progeny phenotypes ranged between and above those of the 803 and GB4 parental lines (Fig. 1C, Table 1), and an inverse relationship was evident between the individual parasite responses in the two assays (Fig. 1D). The two assays yielded a strong negative coefficient of correlation by Spearman’s rank calculation (r = -0.87, 95% CI: -0.95 to -0.72, p < 0.001; Table 2).

Analysis of results from the 803 x GB4 parents and progeny showed that the EC_{50} of LUM were positively and strongly correlated with those of MEF and HLF (r = 0.85, 95% CI: 0.67 to 0.93, p < 0.001; and r = 0.79, 95% CI: 0.56 to 0.91, p < 0.001, respectively; Table 2), consistent with such correlations in a number of previous studies (Basco et al., 1998; Pradines et al., 2006; Basco and Ringwald, 2007; Eyase et al., 2013). This analysis found no correlation between the EC_{50} of LUM and CQ (r = 0.00, 95% CI: -0.42 to 0.41, p = 0.99), in agreement with the results of several reports (Basco et al., 1998; Pradines et al., 1999; Nsobya et al., 2010) but not others that identified a moderate inverse LUM-CQ

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Table 1
Summary of antimalarial drug phenotypes and pfmdr1, pfcr, and pfk13 haplotypes in the 803 x GB4 cross.

| Clones | Haplotype** | pfmdr1 | pfcr | pfk13 | EC_{50} LUM | DTR | EC_{50} MEF | EC_{50} HLF | EC_{50} CQ | EC_{50} DHA |
|--------|-------------|--------|------|-------|------------|-----|------------|------------|------------|------------|
| 803    | 1 1 1       | 2.31   | 3.8  | 31.67 | 1.48       | 147.9 | 74.0       | 1.83       |
| GB4    | 2 2 2       | 0.55   | 17.0 | 10.84 | 0.32       | 88.8  | 5.16       |
| 48C1   | 2 2 2       | 0.69   | 18.0 | 9.31  | 0.32       | 88.8  | 5.16       |
| 36F1   | 2 2 2       | 0.76   | 15.8 | 7.82  | 1.48       | 89.3  | 2.70       |
| 39C3   | 2 2 2       | 0.76   | 18.3 | 7.76  | 0.49       | 47.1  | 1.59       |
| 43A6   | 2 2 2       | 0.76   | 14.0 | 9.03  | 0.53       | 133.3 | 2.23       |
| 46G9   | 2 2 2       | 0.76   | 21.3 | 10.44 | 0.42       | 70.5  | 0.87       |
| 11C2   | 2 2 2       | 0.78   | 19.2 | 7.99  | 0.83       | 67.9  | 1.80       |
| 44F6   | 2 2 2       | 0.82   | 14.0 | 11.05 | 0.59       | 62.2  | ND         |
| 61D3   | 2 2 2       | 0.97   | 12.4 | 11.23 | 1.96       | 49.5  | 1.67       |
| 76H10  | 2 2 2       | 0.97   | 15.0 | 10.83 | 0.87       | 147.1 | 3.74       |
| 37D9   | 2 2 2       | 1.13   | 14.0 | 14.94 | 0.68       | 112.2 | 1.39       |
| 44D4   | 2 2 2       | 1.24   | 19.0 | 8.90  | 1.03       | 111.4 | 2.60       |
| 24G11  | 2 2 2       | 1.49   | 14.0 | 8.85  | 2.38       | 152.8 | 1.68       |
| 36H9   | 2 2 2       | 1.51   | 7.0  | 18.25 | 0.41       | 50.0  | 3.32       |
| 36D5   | 1 2 2       | 1.83   | 10.3 | 22.95 | 1.52       | 132.3 | 2.06       |
| 38G5   | 1 1 1       | 2.69   | 5.7  | 28.72 | 2.64       | 99.1  | 4.35       |
| 39H5   | 1 2 2       | 2.73   | 8.8  | 21.91 | 0.95       | 52.8  | ND         |
| 87E7   | 1 1 1       | 2.79   | 8.3  | 19.64 | 1.38       | 69.7  | 0.85       |
| 36E5   | 1 2 2       | 2.94   | 8.0  | 31.11 | 2.11       | 92.0  | 3.03       |
| 40E7   | 1 1 1       | 3.07   | 7.5  | 33.88 | 1.99       | 93.3  | 1.73       |
| 43E5   | 1 2 2       | 3.30   | 4.8  | 41.13 | 3.10       | 42.2  | 2.22       |
| 39C5   | 1 2 2       | 4.19   | 5.5  | 29.89 | 3.78       | 60.0  | 1.39       |
| 85G7   | 1 1 1       | 5.21   | 4.3  | 47.08 | 3.30       | 78.2  | 1.33       |
correlation (Mwai et al., 2009; Eyase et al., 2013). Finally, our analysis of the EC50 of LUM and those of DHA (determined by S. T. Windle et al. (2018)) showed that these drug responses were uncorrelated (r = −0.12, 95% CI: −0.52 to 0.33, p = 0.61; Table 2); this result agrees with previous findings of no LUM-DHA correlation by Nsobya et al. (2010) and Amambua-Ngwa et al. (2017), although it differs from the weak positive LUM-DHA correlations reported from some other studies (Pradines et al., 2006; Basco and Ringwald, 2007; Mwai et al., 2009).

3.2. Polymorphisms identified by quantitative trait loci (QTL) analysis and correlation tests with genes known to affect drug responses

QTL primary scans of LUM EC50 and DTR phenotypes identified a major peak on Chromosome 5 with maximal LOD scores of 8.94 (p < 0.005) and 8.55 (p < 0.005) respectively (Fig. 2A and B). This peak spans a ~500 kilobase region between the locations of SNPs 702,055 and 1,171,094 of Chromosome 5, with the maximum likelihood location at 966,290. Searches of the PlasmoDB database identified 117 annotated genes within these regions, notably including pfmdr1 at location 957,890 to 962,149 (PF3D7_0523000; Table S3), a candidate gene previously associated with LUM response variations ex vivo, in vivo, and in vitro (Duraishingh et al., 2000; Nzila et al., 2012; Venkatesan et al., 2014; Veiga et al., 2016).

QTL analysis of the MEF EC50 phenotypes likewise identified a primary peak at the pfmdr1 locus (Fig. S1A; p < 0.005). However, scans of the HLF and CQ EC50 phenotypes identified no peaks of statistical significance (Figs. S1B and C; p = 0.34; and p = 0.82 for HLF and CQ, respectively). The absence of a strong pfcr signal with the CQ EC50 values is consistent with the fact the 803 and GB4 parents both have ‘CVIET’ pfcr alleles carrying the 76T codon determinant of CQ resistance (Table 3) that can be modulated by the effects of other genes, including pfmdr1 (Sá et al., 2009; Dhandra et al., 2019).

Using the reported 803 and GB4 genome sequences (Garimella et al., 2020) and copy number analysis, we checked for presence pfmdr1 polymorphisms including the codon variations that are known to occur in other P. falciparum lines at positions 86, 184, 1034, 1042, and 1246 (Foote et al., 1990). A pfmdr1 copy number of 1 was obtained for both GB4 and 803, the same as for the control 3D7 line (Table S4). Relative to the pfmdr1 canonical 3D7 sequence (‘wild-type’), mutant codons 86Y and 184F were identified in the GB4 sequence, whereas all five codons in the 803 sequence matched those of the 3D7 reference sequence (Table 3). The PfMDR1 haplotypes in the 803 × GB4 cross thus differ only at two sites in the 803 (N86/Y184) and GB4 (86Y/184F) parents; accordingly, the results of this study are silent on other combinations of PfMDR1 polymorphisms. While further investigations will be needed to clarify the drug response phenotypes of these other combinations, the linkage of PfMDR1 N86 to the less sensitive LUM and MEF phenotypes of 803 relative to GB4 is consistent with previous reports associating N86 with reduced susceptibility to these drugs (Duraishingh et al., 2000; Sisowath et al., 2005; Dokomajilar et al., 2006; Mwai et al., 2009; Lekana-Douki et al., 2011; Li et al., 2014; Wurtz et al., 2014). The effect of the PfMDR1 Y184F polymorphism on LUM response in association with N86 is less clear: PfMDR1 N86 and Y184 were selected in resistant populations before treatment (Achieng et al., 2015). To our knowledge, the prevalence of Y184 allele was found to be increased in parasite populations post-AL treatment compared to pretreatment samples in coastal Kenya, a slight increase of PfMDR1 184F in as well accounted for the prevalence of Y184 allele in new infections after AL treatment in a Zanzibar cohort (Sisowath et al., 2005; Dokomajilar et al., 2006; Mwai et al., 2009; Lekana-Douki et al., 2011; Li et al., 2014; Wurtz et al., 2014). The effect of the PfMDR1 Y184F polymorphism on LUM response in association with N86 is less clear: PfMDR1 N86 and Y184 were selected in re-infected individuals in new infections after AL treatment in studies in Burkina Faso (Zongo et al., 2007; Some et al., 2010), but PfMDR1 N86 and 184F were more prevalent in new infections after AL treatment in a Zanzibar cohort (Sisowath et al., 2007). In coastal Kenya, a slight increase of PfMDR1 184F in association with N86 was less clear: PfMDR1 N86 and Y184 were selected in re-infections after AL treatment in studies in Burkina Faso (Zongo et al., 2007; Some et al., 2010), but PfMDR1 N86 and 184F were more prevalent in new infections after AL treatment in a Zanzibar cohort (Sisowath et al., 2007). In coastal Kenya, a slight increase of PfMDR1 184F in association with N86 was less clear: PfMDR1 N86 and Y184 were selected in re-infections after AL treatment in studies in Burkina Faso (Zongo et al., 2007; Some et al., 2010), but PfMDR1 N86 and 184F were more prevalent in new infections after AL treatment in a Zanzibar cohort (Sisowath et al., 2007). In coastal Kenya, a slight increase of PfMDR1 184F in association with N86 was less clear: PfMDR1 N86 and Y184 were selected in re-infections after AL treatment in studies in Burkina Faso (Zongo et al., 2007; Some et al., 2010), but PfMDR1 N86 and 184F were more prevalent in new infections after AL treatment in a Zanzibar cohort (Sisowath et al., 2007).
803 × GB4 cross identified no loci in addition to pfmdr1 by scans with genome microarrays, we note that mutations in candidate genes including pfcrt codons N326S and I356T and pfk13 codon C580Y (all of which occur in the 803 parent; Table 3) have been associated with multigenic backgrounds that mediate parasite fitness and susceptibility to a variety of antimalarials (Dhingra et al., 2019). In previous studies of the 803 × GB4 cross, we showed that the PfK13 polymorphism C580Y is linked to survival rates of dihydroartemisinin (DHA)-exposed rings but that it does not determine EC50 levels with this drug (Sa et al., 2018). Tests for correlation of the pfmdr1 haplotype with those of pfcrt or pfk13 alleles did not yield significance at the p < 0.05 level (Spearman rank coefficients r = 0.32, 95% CI: −0.10 to 0.65, p = 0.12; and r = 0.34, 95% CI: −0.08 to 0.66, p = 0.10; Table 2). However, the correlation tests suggested an association of the mutant 326S and 356T codons in the 803

![Fig. 2. Quantitative trait loci (QTL) analysis of lumefantrine phenotypes in the 803 × GB4 cross.](image)

**Table 3**

|        | PI MDR1 | PCRT | PI CRT | PK13 |
|--------|---------|------|--------|------|
| Parasite line | 86 | 184 | 1034 | 1246 | copyno | 72 | 74 | 75 | 76 | 220 | 271 | 326 | 356 | 371 | 189 | 580 |
| 3D7    | N | Y | S | N | D | I | C | M | N | K | A | Q | N | I | R | K | C |
| 803    | N | Y | S | N | D | I | C | I | E | T | S | E | S | T | I | K | Y |
| GB4    | Y | F | S | N | D | I | C | I | E | T | S | E | N | I | I | T | C |

*Details of copy number analysis are provided in Table S4.*
the PfK13 propeller mutations and their association with treatment failures.

4. Conclusions

LUM is a vital partner in what is perhaps the leading ACT worldwide for the treatment of uncomplicated *P. falciparum* malaria, and MEF continues to be widely used in ACTs by a number of countries in South American and Southeast Asia, including Cambodia (World Health Organization, 2019). In this study, the results from two different drug response assays with LUM, a 72 h EC₅₀ growth inhibition assay and a DTR parasitemia recovery assay following 24 h drug exposure, showed strong inverse correlation in the parents and 22 independent recombinant progeny of a cross between Cambodian (803) and Ghanaian (GB4) lines of *P. falciparum*. Strong correlations were also found between LUM, MEF, and HLF susceptibilities in the 803 × GB4 cross. By QTL analysis and Mann-Whitney U tests with candidate genes known to be involved in heme metabolism, LUM, MEF, and HLF phenotypes were associated with inheritance of PfMDR1, confirming previous findings that the wild-type N86 residue confers decreased susceptibility to these drugs. Statistical tests likewise identified moderately reduced CQ susceptibility from mutations N326S and I356T in the 803 version of the CVIET-type PfCRT carried by both parents; however, the well-known reduction of LUM susceptibility from wild-type PfCRT K76 could not be assessed as all parents and progeny contained PfCRT 76T. Finally, statistical tests of PfK13 Kelch protein polymorphisms identified significant reductions of LUM and MEF susceptibility with inheritance of the PfK13 580Y mutation in the 803 parent. In endemic regions where LUM and MEF are used in ACTs, pressure on parasite populations from these partner drugs may boost the prevalence of PfK13 580Y and other Kelch polymorphisms involved in drug susceptibility.

Ethics approval and consent to participate

Not applicable.

Availability of data and materials

All data generated or analyzed in this study are provided in the main text of this article or as supplementary information in supporting files.

Authors’ contributions

STW, KDL, NBG, and TEW designed the study and wrote the manuscript. STW, KDL, NBG, AL, and JMS collected data, STW, KDL, JM, RLC, RSR, JMS, and TEW contributed resources and analyzed results. All authors read, revised, and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no competing interests

Acknowledgements

The authors thank Christine E. Figan for her assistance in microsatellite genotyping, Kiran V. Garimella for providing PacBio DNA sequences for the parental lines, and Michael P. Fay for discussion of statistical correlation tests. This work was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Disease, National Institutes of Health.
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Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2020.10.009.
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