Inhibitory Activity of Ferroquine, versus Chloroquine, against western Kenya *Plasmodium falciparum* Field Isolates Determined by a SYBR Green I *In Vitro* Assay

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Abstract. Ferroquine (FQ), a chloroquine (CQ) analog, is being developed to treat persons with *Plasmodium falciparum* malaria. In 146 *P. falciparum* field isolates from western Kenya, we measured 50% inhibitory concentrations (IC_{50} ; nM) of CQ and FQ by a SYBR Green I *in vitro* assay. Reference clones included W2 (CQ resistant) and D6 (CQ sensitive). Mutation analysis was done for *P. falciparum* CQ-resistance transporter gene (*Pfcr*t K76T). Median IC_{50} values for FQ were lower than CQ for field isolates and the W2 clone (both $P < 0.05$). The *Pfcr*t mutation (76T), which was detected in > 80% of isolates, conferred higher CQ IC_{50} values ($P < 0.05$) and modestly lower FQ IC_{50} values ($P < 0.05$), versus *Pfcr*t wild type (K76). FQ is more potent than CQ against CQ-resistant *P. falciparum* field isolates and the W2 clone, and is less affected by *Pfcr*t 76T. These findings support the notion that FQ could be useful in treating persons with *P. falciparum* malaria.

INTRODUCTION

Ferroquine (FQ; SSR 97193) is a new 4-aminoquinoline organometallic chloroquine (CQ) analog that is being developed for the curative treatment of adults and children with uncomplicated malaria caused by *Plasmodium falciparum*, likely in multi-drug regimens with an artemisinin. *In vitro* data indicate that FQ is active against CQ-resistant and CQ-sensitive *P. falciparum* strains and against *P. vivax*.¹

An increasing number of reports describe *in vitro* growth inhibitory capability of FQ against CQ-resistant and CQ-sensitive *P. falciparum* laboratory strains, often superior to CQ.^{2–8} As clinical trials of FQ proceed in support of licensure, additional *in vitro* testing of *P. falciparum* field isolates, especially those obtained from sites of FQ efficacy trials with known CQ-resistant *P. falciparum*, may improve understanding of the capabilities of FQ. In Kenya, where drug resistance often emerges in eastern Africa, *P. falciparum* remains largely resistant to CQ, which suggests a useful location to obtain isolates for *in vitro* testing.^{9–11}

The aim of this study was to assess the inhibitory activity of CQ versus FQ against *P. falciparum* field isolates obtained in western Kenya, a region where CQ resistance is well established. To do this assessment, immediate *ex vivo* (IEV) or culture-adapted *P. falciparum* field isolates were examined *in vitro* by using a SYBR Green I 50% inhibitory concentration (IC_{50}) drug sensitivity assay. Additionally, we assessed *P. falciparum* field isolates for drug resistance markers (*P. falciparum* multidrug resistance 1 [*Pfmdr*1] gene and *P. falciparum* chloroquine resistance transporter [*Pfcr*t]) gene to find associations with IC_{50} values.

MATERIALS AND METHODS

Protocol and sites. The study was approved by the Kenya Medical Research Institute and the Walter Reed Army Institute of Research institutional review boards (protocol nos. KEMRI 1330 and WRAIR 1384).

Participating centers, all of which were Kenya Ministry of Health facilities in western Kenya, included Kisumu, Kisii, and Kericho District Hospitals. Kisumu is a lowland, malaria holoendemic area, and Kisii and Kericho are highland, malaria hypoendemic areas.¹² Persons attending outpatient clinics in 2009–2010 who were ≥ 6 months of age and had suspected uncomplicated *P. falciparum* malaria were invited to participate in the study. Written informed consent was obtained from all participants or guardians. Anyone treated for malaria within the past one month was ineligible.

Sample collection and preparation. Blood samples (2–3 mL) were obtained from persons who consented and had a positive *P. falciparum* rapid diagnostic test (RDT) (Parascreen; Zephyr Biomedicals, Verna Goa, India) and transported to the laboratory. Three blood spots (each approximately 100 μ L) were placed on FTA filter paper (Whatman Inc., Bound Brook, NJ) for parasite DNA extraction and molecular analysis. Malaria blood films on glass slides were made for staining with Giemsa in the laboratory for microscopic examination to confirm RDT results and determine parasitemia. For discrepancies between RDT and microscopy results, microscopy results were used as final results.

Plasmodium falciparum-infected blood specimens from Kisumu District Hospital (< a 15 minute drive to the laboratory) were collected in acid citrate dextrose vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ) and transported within four hours for IEV processing. *Plasmodium falciparum*-infected blood specimens from Kericho and Kisii District Hospitals (2-hour drives to the laboratory) were placed in storage-transport medium and refrigerated at 4°C until transported to the laboratory, generally within 72 hours, for culture-adaptation.¹⁰

***In vitro* IC_{50} drug testing.** A SYBR Green I-based *in vitro* IC_{50} drug sensitivity assay was used to test each *P. falciparum* field isolate against CQ and FQ.^{13–16} Ferroquine was provided by Sanofi-Aventis Corporation (Paris, France), and CQ was provided by the Walter Reed Army Institute of Research (Silver Spring, MD).

Plasmodium falciparum laboratory reference clones assayed periodically for internal controls against both drugs included D6, classified as CQ sensitive, and W2, classified as CQ resistant.¹⁷ Clones D6 and W2 were obtained from frozen stocks and culture-adapted for SYBR Green I assays.

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Stock drug solutions at concentrations of 1 mg/mL were prepared. Ferroquine was dissolved in absolute ethanol, and CQ was dissolved in 1.5 mL of de-ionized water and 3.5 mL of absolute ethanol. Further dilutions were in complete RPMI 1640 medium to the desired starting concentration, followed by serial two-fold dilutions to generate 10 concentrations across the plates. Dilution ranges were as follows: CQ (3,125–6 nM) and FQ (2,305–4.5 nM). Pre-diluted drug plates were prepared fresh and used immediately or stored at -80°C for not longer than one month before use.

Plasmodium falciparum isolates processed IEV were assayed without culture adaptation.¹⁶ Blood samples with $>1\%$ parasitemias were adjusted to 1% parasitemias at 2% hematocrits, and those with $\leq 1\%$ parasitemias were used unadjusted at 2% hematocrits. For IEV and culture-adapted assays, transfer of parasite samples and drug aliquots onto microculture plates and IC_{50} values depicted by the SYBR Green I drug assay were as described.¹⁶

Plasmodium falciparum field isolates obtained from two sites were culture adapted before assay. The isolates were cultured at a 6% hematocrit to establish parasite replication robustness, reaching $3\text{--}8\%$ parasitemia within $7\text{--}30$ days.^{16,17} For IC_{50} drug assays, *P. falciparum* aliquots were adjusted to a 2% hematocrit and a 1% parasitemia.

Molecular analyses of *P. falciparum* field isolates and reference clones. *Plasmodium falciparum* field isolate DNA for all molecular assays was extracted from FTA filter paper samples or whole blood by using the QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA) according to manufacturer's instructions. Genomic DNA from laboratory clones used as controls was extracted in similar fashion.

A single nucleotide polymorphism (SNP), K76T, in the *Pfprt* gene, which is well described in Kenya in conferring CQ resistance, was determined as described.¹⁰ For *Pfmdr1*, an estimated copy number was determined by using a $2^{-\Delta\Delta\text{Ct}}$ method of relative quantification.¹⁸ For the calibrator, we used *P. falciparum* reference clone 3D7 genomic DNA that contained one copy of *Pfmdr1*. *Plasmodium falciparum* tubulin was used as a housekeeping gene, and Dd2 laboratory reference clone DNA was used as a *Pfmdr1* multiple gene copy control. The SNP analysis for *Pfmdr1* codons 86, 184, 1034, and 1042 was conducted as described^{19,20} by using a real-time, mutation-specific polymerase chain reaction.

Statistical analyses. Data were analyzed by using non-parametric Kruskal-Wallis one-way analysis of variance by ranks, the Mann-Whitney U test, Dunn's multiple comparison test, and Pearson product-moment correlation coefficient (GraphPad Prism 4.00 for Windows; GraphPad Software, San Diego, CA, and Sigma Plot version 10; Systat Software, Inc., San Jose, CA).

RESULTS

SYBR Green I IC_{50} drug assays. We collected 146 *P. falciparum* field isolates and assayed 88 IEV and 58 as culture-adapted against CQ and FQ by using a SYBR Green I assay. A successful assay result was defined as a dose-response relationship across the 10 drug dilutions, for one or both drugs, per field isolate.

Individual and median IC_{50} values (nM) for IEV and culture-adapted *P. falciparum* field isolates against CQ and FQ and *P. falciparum* laboratory reference clones D6 (CQ sensi-

FIGURE 1. Chloroquine (CQ) and ferroquine (FQ) SYBR Green I *in vitro* drug 50% inhibitory concentration (IC_{50}) assays for *Plasmodium falciparum* field isolates and *P. falciparum* laboratory reference clones D6 and W2 (right). Individual and median values (closed and open symbols, blue and red bars [online version], and black and gray bars [print version]), respectively are shown. IEV = immediate *ex vivo*; CA = culture-adapted. Comparison of CQ versus FQ medians within three subsets: *P. falciparum* field isolates IEV Kisumu, CA Kisii-Kericho, and *P. falciparum* reference clones D6 versus W2 (all P values < 0.05 by Mann-Whitney rank sum test). Black solid line at 141 nM indicates CQ IC_{50} historical breakpoint value for ^3H -hypoxanthine assays in *P. falciparum* field isolates, which is considered discriminative for resistance *in vivo*.

tive) and W2 (CQ resistant) are shown in Figure 1. Median IC_{50} values for CQ were consistently higher than those for FQ for IEV and culture-adapted isolates. For CQ IC_{50} values, 17 (21%) of 81 IEV and 12 (21%) of 58 culture-adapted *P. falciparum* isolates had values > 141 nM, which is a previously established discriminative IC_{50} breakpoint value indicative of CQ resistance.¹⁰ For FQ, there is no discriminative IC_{50} value breakpoint indicative of resistance. We observed no difference between FQ median IC_{50} values for IEV or culture-adapted isolates versus those of D6 CQ-sensitive and W2 CQ-resistant laboratory reference clones ($P > 0.05$).

A Pearson correlation coefficient analysis of CQ and FQ IC_{50} values was performed for IEV and culture-adapted isolates. The r values of -0.001178 and -0.0596 for IEV and culture-adapted isolates, respectively, are shown in Figure 2. These values indicated a lack of correlation (cross-resistance) between CQ and FQ *in vitro* activity.

For *P. falciparum* reference clones D6 (CQ sensitive) and W2 (CQ resistant), the median CQ IC_{50} value for each clone was greater than that for FQ ($P \leq 0.05$). The CQ IC_{50} median value against W2 was higher than the 141 nM breakpoint value.

Molecular assays of *P. falciparum* field isolates and laboratory reference clones. The *Pfprt* mutation 76T , which is present in $> 80\%$ (87 of 108) of *P. falciparum* field isolates, conferred higher median IC_{50} values for CQ, but not for FQ, in comparison with *Pfprt* wild type 76T (Figure 3A and B). For FQ, the *Pfprt* genotype had little effect on median IC_{50} values (Figure 3B). Among all *P. falciparum* field isolates expressing *Pfprt* mutation 76T , the median IC_{50} value for CQ was higher than that for FQ ($P < 0.05$, by Mann-Whitney U test). As in earlier work, mixed (wild type plus mutant) and mutant *Pfprt*

FIGURE 2. Pearson's correlation coefficients of the log 50% inhibitory concentration (IC_{50}) values of chloroquine (CQ)-immediate *ex vivo* (IEV) versus ferroquine (FQ)-IEV values, and CQ-culture-adapted (CA) versus FQ-CA values. Values were -0.001178 and -0.0596 , respectively, which indicated no association (possible cross-resistance) between *in vitro* activity of CQ and FQ.

K76T genotypes were combined in some analyses to express a total mutant population.^{10,16}

The *Pfmdr1* gene was not amplified in *P. falciparum* field isolates when quantified relative to *P. falciparum* reference clones 3D7 (mean = 1 copy) and multiple-drug resistant Dd2 (mean = 3 copies) (Figure 4A). The proportions of *Pfmdr1* codon SNPs were highest at codon 86 (33%). There were far fewer at codons 184 and 1034 and none at codon 1042, regard-

less of collection site. The *Pfmdr1* codon 86 genotype (wild type versus mutant) expressed in *P. falciparum* field isolates had little effect on CQ or FQ median IC_{50} values (Figure 4B).

DISCUSSION

In a direct comparison of antimalarial activities of CQ and FQ by using an SYBR Green I *in vitro* assay, we found that FQ was consistently more potent than CQ against *P. falciparum* field isolates obtained in western Kenya, a region to which CQ-resistant *P. falciparum* is endemic. Moreover, FQ showed higher potency than CQ against the well-characterized CQ-resistant *P. falciparum* laboratory clone W2. As expected, most *P. falciparum* field isolates expressed the *Pfcr1* mutation (76T), which conferred higher median IC_{50} values for CQ but, notably, not for FQ. Our results indicate that FQ should be used to treat persons with *P. falciparum* malaria, even persons infected with CQ-resistant strains.

Superior *in vitro* activity of FQ than of CQ has been reported.²¹⁻²³ Our study extends observations of FQ superiority against recently collected CQ-resistant *P. falciparum* field isolates and the CQ-resistant *P. falciparum* laboratory reference clone W2. The superiority of FQ was evident in field isolates processed IEV or by culture adaptation. The immediate *ex vivo* procedure is a simpler and faster technique that in comparison with culture adaptation, reduces cold storage and processing steps of *P. falciparum* isolates and may reduce clonal selection.^{13,24}

Median CQ IC_{50} values in the SYBR Green I assay for culture-adapted and IEV samples generally paralleled our earlier ³H-hypoxanthine uptake findings in culture-adapted

FIGURE 3. **A**, Chloroquine (CQ) and **B**, ferroquine (FQ) 50% inhibitory concentration (IC_{50}) values, respectively, plotted against *Plasmodium falciparum* chloroquine resistance transporter (*Pfcr1*) gene wild, mixed, and mutant genotypes. Individual and median 76T values (closed symbols/black bars, and open symbols/gray bars, respectively) are shown. **a**, *P. falciparum* field isolates expressing *Pfcr1* mutation (K76) associated with higher CQ IC_{50} median values (wild versus mixed versus mutant; $P < 0.05$, by Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks; wild versus mutant; $P < 0.05$, by Dunn's method). **b**, *P. falciparum* field isolate *Pfcr1* genotype showed little effect on FQ IC_{50} median values (gray bars); (wild 76T versus mixed versus mutant; $P > 0.05$, by Kruskal-Wallis one way ANOVA on ranks). **c**, *P. falciparum* field isolates expressing the *Pfcr1* mutation (K76) associated with higher median IC_{50} for CQ, versus FQ (closed symbols/black bar and open symbols/gray bar, respectively); ($P < 0.05$, by Mann-Whitney rank sum test).

FIGURE 4. **A**, *Plasmodium falciparum* multidrug resistance 1 (*Pfmdr1*) gene copy number in *P. falciparum* field isolates and two reference clones. Individual and median values (gray bars) are shown. Several *P. falciparum* field isolates with slightly higher copy number expression (outliers) were from different sites and had chloroquine (CQ) and ferroquine (FQ) 50% inhibitory concentration (IC₅₀) values within their respective group range. **B**, CQ and FQ IC₅₀ respectively, plotted against *Pfmdr1* codon 86 wild versus mutant genotypes. Individual and median values (closed symbols/black bars, open symbols/gray bars) are shown. For CQ and FQ, *Pfmdr1* codon 86 genotype (wild versus mutant) had little effect on IC₅₀ median values ($P > 0.05$, by Mann-Whitney U test, for CQ [wild versus mutant] and FQ [wild versus mutant]).

P. falciparum isolates from Kenya.¹⁰ For example, approximately 21% of CQ IC₅₀ values determined by SYBR Green I assay exceeded a previously established discriminative value of 141 nM, and CQ IC₅₀ value ranges for the *P. falciparum* laboratory reference clones D6 and W2 were in accordance with established CQ sensitivity profiles.¹⁰ We suggest that these data, especially because there is no *in vitro* discriminative resistance value for FQ resistance, can be used as a modest baseline from which to track and compare FQ IC₅₀ values, especially in eastern Africa.

When CQ and FQ IC₅₀ values for *P. falciparum* field isolates were plotted against those for specific *Pfcr* genotypes, we found a direct relationship with CQ (higher median IC₅₀ value with *Pfcr* mutation 76T) and an inverse relationship with FQ (lower median IC₅₀ value with *Pfcr* wild-type gene K76). The former result was expected, given the known prevalence of *Pfcr* 76T in *P. falciparum* isolates in Kenya. The latter result

suggested that *Pfcr* 76T has a minimal effect on FQ activity.⁵ Ferroquine, which is an organo-metallic complex, differs from CQ in diverse ways, including lipophilicity, shape, volume, and electronic profile.^{22,23} These differences facilitate FQ entry into the *P. falciparum* vacuoles and, as some investigators propose, prevent FQ extrusion conferred by the *Pfcr* 76T mutation, which characterizes CQ-resistant strains.^{5,6,21} The ferrocene moiety may confer some of the properties of FQ.²⁵ In parallel with these earlier findings, our study found no association between IC₅₀ values for CQ and FQ, which suggested no cross-resistance.

The *Pfmdr1* gene confers multiple drug resistance when more than one copy is present and is common in Southeast Asia.²⁶ As expected, Kenya *P. falciparum* field isolates showed no amplification of the *Pfmdr1* gene, which is consistent with findings for the wild type, and are not necessarily associated with multi-drug resistance capability.^{27,28} This finding precluded comment on a role of the *Pfmdr1* gene copy number on FQ activity. The *Pfmdr1* codon 86 genotype had relatively little effect on CQ and FQ IC₅₀ values.^{7,8} Nonetheless, it may be useful to track FQ IC₅₀ values versus *Pfmdr1* codon 86 genotypes because *P. falciparum* FQ resistance markers are not fully identified.

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