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Ex Vivo Drug Susceptibility of Ferroquine against Chloroquine-Resistant Isolates of *Plasmodium falciparum* and *P. vivax*▽

Jutta Marfurt,[1]* Ferryanto Chalfein,[2] Pak Prayoga,[2] Frans Wabiser,[2] Enny Kenangalem,[2,3] Kim A. Piera,[1] Barbara MacHunter,[1] Emiliana Tjitra,[4] Nicholas M. Anstey,[1] and Ric N. Price[1,5]

Global Health Division, Menzies School of Health Research, Charles Darwin University, Darwin, Australia[1]; Menzies School of Health Research—National Institute of Health Research and Development Malaria Research Program, Timika, Indonesia[2]; District Ministry of Health, Timika, Papua, Indonesia[3]; National Institute of Health Research and Development, Ministry of Health, Jakarta, Indonesia[4]; and Centre for Vaccinology & Tropical Medicine, Nuffield Department of Clinical Medicine, John Radcliffe Hospital, Oxford, United Kingdom[5]

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Ferroquine (FQ; SSR97193), a ferrocene-containing 4-aminoquinoline derivate, has potent *in vitro* efficacy against chloroquine (CQ)-resistant *Plasmodium falciparum* and CQ-sensitive *P. vivax*. In the current study, *ex vivo* FQ activity was tested in multidrug-resistant *P. falciparum* and *P. vivax* field isolates using a schizont maturation assay. Although FQ showed excellent activity against CQ-sensitive and -resistant *P. falciparum* and *P. vivax* (median 50% inhibitory concentrations [IC_{50}s], 9.6 nM and 18.8 nM, respectively), there was significant cross-susceptibility with the quinoline-based drugs chloroquine, amodiaquine, and piperaquine (for *P. falciparum*, $r = 0.546$ to 0.700, $P < 0.001$; for *P. vivax*, $r = 0.677$ to 0.821, $P < 0.001$). The observed *ex vivo* cross-susceptibility is likely to reflect similar mechanisms of drug uptake/efflux and modes of drug action of this drug class. However, the potent activity of FQ against resistant isolates of both *P. falciparum* and *P. vivax* highlights a promising role for FQ as a lead antimalarial against CQ-resistant *Plasmodium* and a useful partner drug for artemisinin-based combination therapy.

The public health importance of *Plasmodium vivax* has been neglected despite causing an estimated 70 to 391 million clinical infections each year (2, 20, 24). Once regarded as a benign infection, there is increasing evidence that vivax malaria is an important cause of morbidity and developmental impairment, inflicting a huge socioeconomic burden on countries of disease endemicity (3, 20). In addition, recent reports about severe and fatal vivax malaria (15, 30) raise the possibility of an emerging threat of severe disease associated with partially effective treatment (23). Chloroquine (CQ)-resistant (CQR) *P. vivax* is now widespread across much of the areas of malaria endemicity, and there are calls for artemisinin-based combination therapy (ACT) to be deployed for both *P. falciparum* and *P. vivax* (14). In areas of coendemicity of *P. falciparum* and *P. viva*, this treatment approach combines the fast-acting artemisinin compound with a partner drug with a longer half-life, such as 4-aminoquinolines, that have a proven efficacy in both species.

Ferroquine (FQ; SSR97193) is an organometallic drug which contains a ferrocenyl group covalently flanked by a 4-aminoquinoline and a basic alkylamine. Among the more than 100 ferrocene analogues synthesized and screened to date (7, 8, 13), FQ proved to be the best antimalarial candidate. *In vivo* experiments performed on rodent *Plasmodium* species demonstrated powerful activity of the drug and a high oral bioavailability, two major qualities for selecting lead compounds for further development (6, 11). *In vitro* susceptibility of FQ has been tested in different culture-adapted *P. falciparum* strains (5, 11, 12) and field isolates from Gabon, Africa, Senegal, Cambodia, and Thailand (1, 4, 9, 21, 22) and has proven to be effective against CQ-sensitive (CQS) and CQR strains. More recently, the *ex vivo* activity of FQ against chloroquine-sensitive strains of *P. vivax* has been demonstrated in Thailand (19).

In several studies, weak cross-reactivity with CQ has been described in *P. falciparum* isolates (1, 4, 9, 21, 22). However, other studies have not observed significant correlation of *in vitro* drug responses between FQ and other standard drugs (16, 19).

The objectives of the current study were to examine the species-specific *ex vivo* susceptibility of FQ in clinical isolates of *P. falciparum* and *P. vivax* from an area with known multidrug resistance in both species and to investigate cross-susceptibility patterns with conventional antimalarials CQ, amodiaquine (AQ), and piperaquine (PIP).

Plasmodium isolates were collected between September 2008 and December 2009 from patients attending malaria clinics in Timika, Papua Province, Indonesia, a region where multidrug-resistant strains of *P. vivax* and *P. falciparum* are endemic (17, 25, 26, 29). Patients were recruited into the study if presenting with symptomatic malaria and single infection with *P. falciparum* or *P. vivax*, with a parasitemia of between 2,000 μl^{-1} and 80,000 μl^{-1}, and a majority (>50%) of parasites at the ring stage of development. Patients treated with antimalarials in the past month were excluded from this study. Ve-

* Corresponding author. Mailing address: Menzies School of Health Research, P.O. Box 41096, Casuarina, NT 0811, Darwin, Australia. Phone: 61 (0)8 8922 8197. Fax: 61 (0)8 8922 8429. E-mail: Jutta.Marfurt@menzies.edu.au.

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TABLE 1. Baseline characteristics of isolates for which *ex vivo* assay was accomplished

Baseline characteristic[a]	Value for:	
	P. falciparum	*P. vivax*
Total no. of isolates reaching harvest/total no. of isolates (%)	50/59 (85)	51/59 (86)
Median delay (range) from venepuncture to start of culture (min)	122.5 (45–246)	111 (35–200)
Median duration (range) of assay (h)	46 (32–56)	47 (41–50)
Geometric mean parasitemia (no. of asexual parasites/μl) (95% CI)	13,582 (9,800–18,822)	9,914 (7,739–12,700)
Mean schizont count at harvest (95% CI)	41 (38–44)	38 (34–41)
Median initial % of parasites (range) at ring stage	100 (100–100)	90 (52–100)

[a] CI, confidence interval.

nous blood samples (5 ml) were collected by venepuncture, and after removal of host white blood cells by using CF11 cellulose (27), packed infected red blood cells (IRBC) were used for the *ex vivo* drug susceptibility assay.

Drug susceptibilities of *P. vivax* and *P. falciparum* isolates were measured using a protocol modified from the WHO microtest, as described previously (28). Standard antimalarial drugs CQ, AQ (Sigma-Aldrich, Australia), and PIP (Ranbaxy Lab. Ltd., Gurgaon, India) and experimental compound FQ (Sanofi-Aventis, Paris, France) were prepared as 10 mM stock solutions in dimethyl sulfoxide (DMSO). Drug plates were predosed by diluting the compounds in 50% ethanol followed by lyophilization and stored at 4°C. The drug plate quality control was assessed by defining drug response profiles in CQ-resistant and -sensitive laboratory strains K1 and FC27, respectively, before and after completion of the study.

Two hundred microliters of a 2% hematocrit blood media mixture (BMM), consisting of RPMI 1640 medium plus 10% AB^+ human serum (*P. falciparum*) or McCoy's 5A medium plus 20% AB^+ human serum (*P. vivax*), was added to each well of predosed drug plates containing 11 serial concentrations (2-fold dilutions) of the antimalarials (maximum concentrations shown in parentheses) CQ (2,992 nM), AQ (80 nM), PIP (769 nM), and FQ (590 nM). A candle jar was used to mature the parasites at 37.5°C for 30 to 56 h. Incubation was stopped when >40% of ring-stage parasites had reached the mature schizont stage in the drug-free control well.

Thick blood films made from each well were stained with 5% Giemsa solution for 30 min and examined microscopically. The number of schizonts per 200 asexual stage parasites was determined for each drug concentration and normalized to the control well.

The dose-response data were analyzed using nonlinear regression analysis (WinNonlin 4.1; Pharsight Corporation) and the median 50% inhibitory concentration (IC_{50}) was derived using an inhibitory sigmoid maximum effect (E_{max}) model. Derived IC_{50} data were used only from predicted curves for which the E_{max} and minimum effect (E_0) were within 15% of 100 or 0, respectively.

Analysis was performed using STATA software (version 10.1; Stata Corp., College Station, TX). The Mann-Whitney U test, Wilcoxon signed-rank test, and Spearman rank correlation were used for nonparametric comparisons and correlations; all data were log transformed for parametric analyses of covariance.

Ethical approval for this study was obtained from the ethics committees of the National Institute of Health Research and Development, Ministry of Health, Indonesia, and the Menzies School of Health Research, Darwin, Australia.

Ex vivo susceptibility of FQ (SSR97193) was tested in field isolates obtained from 120 patients presenting with single-species infections of either *P. falciparum* ($n = 60$) or *P. vivax* ($n = 60$). Susceptibility profiles of the same isolates were also tested against CQ, AQ, and PIP. Adequate growth for harvest was achieved in 85% (50/59) of *P. falciparum* isolates and 86% (51/59) of *P. vivax* isolates. Baseline characteristics of the isolates processed are presented in Table 1, and the median IC_{50}s of each drug for the two species are presented in Table 2. Since previous studies have highlighted a major difference in the stage-specific activities of CQ and AQ, the same analysis for *P. vivax* was restricted to isolates with ≥80% of asexual forms at ring stage at the start of the assay (39/51, 76%). However, no statistically significant difference in median IC_{50}s for any of the

TABLE 2. Overall *ex vivo* activity for each drug according to the species tested

Drug	IC_{50} (nM) for *P. falciparum* lab line[a]		*P. falciparum*, clinical field isolates		*P. vivax*, all isolates		*P. vivax*, isolates with ≥80% rings	
	FC27 (CQS)	K1 (CQR)	No. of assays (%)[b]	Median IC_{50} (nM) (range)	No. of assays (%)[b]	Median IC_{50} (nM) (range)	No. of assays (%)[b]	Median IC_{50} (nM) (range)
Chloroquine	28.2	170.5	50/50 (100)	94.9 (7.9–336.1)	50/51 (98)[c]	92.0 (12.9–386.5)	39/39 (100)	97.3 (12.9–386.5)
Amodiaquine	23.1	23.2	35/36 (97)[d]	12.3 (1.1–49.5)	32/32 (100)	16.9 (5.6–51.5)	24/24 (100)	21.0 (5.6–51.5)
Piperaquine	49.4	54.3	50/50 (100)	21.8 (0.3–63.9)	51/51 (100)	23.1 (1.3–93.4)	39/39 (100)	23.4 (1.3–93.4)
Ferroquine	18.6	20.7	44/45 (98)[d]	9.6 (0.6–71.5)	48/48 (100)	18.8 (1.6–48.9)	37/37 (100)	20.4 (1.6–48.9)

[a] Mean IC_{50}s (derived from 2 independent experiments) assessed by *in vitro* schizont maturation and quantified by microscopy. CQS, chloroquine sensitive; CQR, chloroquine resistant.
[b] Total number of assays with acceptable IC_{50}s/total number of assays harvested (%).
[c] One highly resistant *P. vivax* isolate (i.e., model was not possible).
[d] Model not possible for 1 *P. falciparum* isolate.

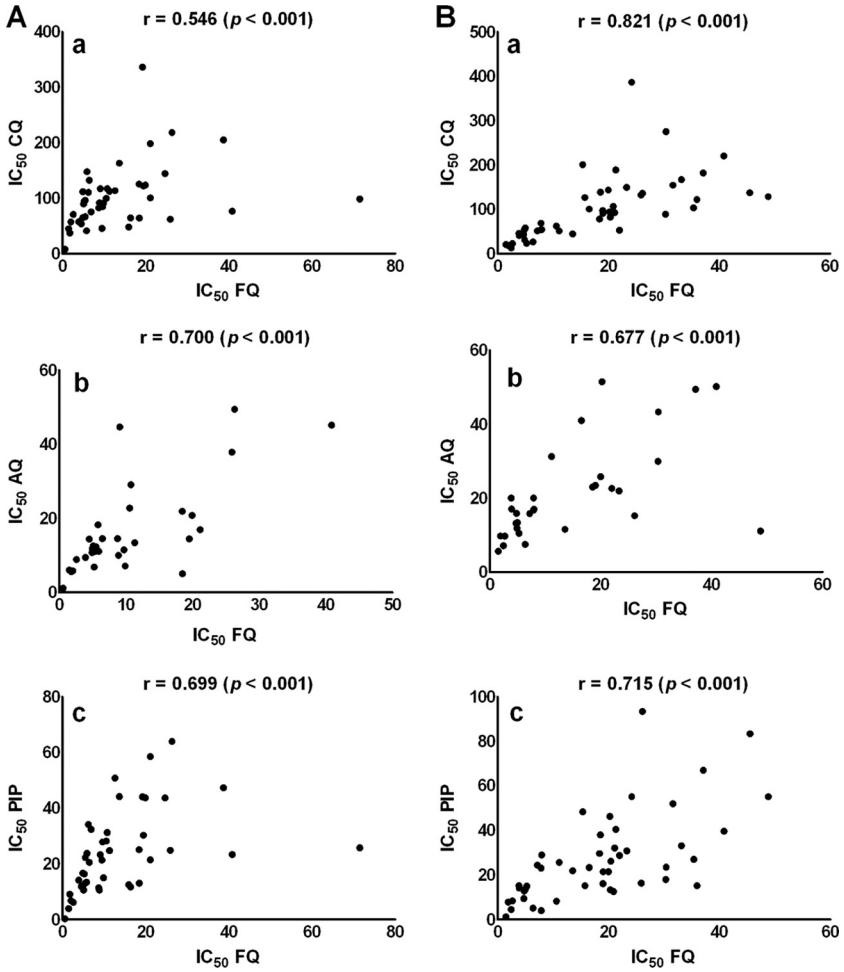

FIG. 1. Correlation of *in vitro* drug susceptibility of FQ and quinoline-based standard antimalarials in multidrug-resistant clinical isolates of *P. falciparum* (A) and *P. vivax* (B) from Papua, Indonesia. FQ, ferroquine; CQ, chloroquine; AQ, amodiaquine; PIP, piperaquine; *r*, Spearman rank correlation coefficient. (a) FQ versus CQ; (b) FQ versus AQ; (c) FQ versus PIP.

drugs tested was found between isolates with ≥80% and 50 to 80% ring stages, respectively.

The median IC_{50} for FQ against *P. vivax* was 18.8 nM (range, 1.6 to 48.9), compared to 9.6 nM against *P. falciparum* (range, 0.6 to 71.5; $P = 0.045$). There was no correlation between the IC_{50} and initial parasitemia for either species. Significant cross-susceptibility was observed between FQ and the quinoline-based drugs CQ, AQ, and PIP for both *P. falciparum* ($r = 0.546$ to 0.700; $P < 0.001$) and *P. vivax* ($r = 0.677$ to 0.821; $P < 0.001$) (Fig. 1), and these correlations remained after controlling for initial parasitemia (18).

Ferroquine is a promising, novel ferrocene 4-aminoquinoline with potent *in vitro* activity against CQ-sensitive (CQS) and -resistant (CQR) *P. falciparum* strains from various geographical areas (4, 9, 18, 21, 22) as well as good *in vivo* efficacy in rodent malaria (6, 11). A recent report of *ex vivo* drug susceptibility of *P. vivax* on the Thai-Burmese border supports the further development of this lead antimalarial compound (19).

In the current study, cross-susceptibility patterns of FQ in clinical isolates of *P. falciparum* and *P. vivax* in an area highly prevalent for multidrug resistance in both species were assessed (17, 25, 26). We observed FQ IC_{50}s in the low nM range for both CQR and CQS *P. falciparum* isolates, in line with previous reports from Africa and Southeast Asia (4, 9, 18, 21, 22). The *ex vivo* activity of FQ against multidrug-resistant *P. vivax* isolates from Papua, Indonesia (median IC_{50} = 18 nM), was comparable to the IC_{50} (median = 15 nM) recently observed against *P. vivax* isolates from the Thai-Burmese border where most isolates are chloroquine sensitive (19). Our study provides data of FQ activity in isolates with a greater range of CQ susceptibility and highlights significant cross-susceptibility between FQ and the conventional quinolines CQ, AQ, and PIP. Our methodology controls for variation in the initial hematocrit but does not control for an inoculum effect which has been reported to confound the *in vitro* assessment of drug susceptibility in *P. falciparum* (18). We found no correlation between initial parasitemia and IC_{50} in either of the species, and the correlations between drugs remained after controlling for initial parasitemia.

The observed cross-susceptibility patterns are consistent with quinoline-based drugs sharing a common mode of action. However, the within-species differences of the median IC_{50}s of CQ, AQ, PIP, and FQ indicate that there are likely to be other mechanisms such as drug uptake, metabolism, and efflux, or

resistance, involved in determining *ex vivo* drug responses to these drugs.

The strong activity of FQ on CQR isolates of *P. falciparum* and *P. vivax* suggests significant differences in the resistance mechanisms of the parasites. For *P. falciparum*, previous studies have shown that FQ activity is independent of known mutations in CQR relevant genes (10, 16, 18). Although FQ-resistant laboratory strains have not yet been produced (10), further studies elucidating the cause of activity of FQ on CQR strains will help to shed light on possible parasite resistance mechanisms to both drugs.

In conclusion, our data showing excellent *ex vivo* activity of FQ against multidrug-resistant isolates of both *P. falciparum* and *P. vivax* further support the potential use of FQ against malaria in regions where both *Plasmodium* species are endemic.

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