In Vitro Activity of Ferroquine Is Independent of Polymorphisms in Transport Protein Genes Implicated in Quinoline Resistance in Plasmodium falciparum

Maud Henry,1,2 Sébastien Briolant,1,2 Albin Fontaine,1,2 Joel Mosnier,1,2 Eric Baret,1,2 Rémy Amalvict,1,2 Thierry Fusaï,1,2 Laurent Fraisse,3 Christophe Rogier,1,2 and Bruno Pradines1,2*

Unité de Recherche en Biologie et Épidémiologie Parasitaires, Institut de Médecine Tropicale du Service de Santé des Armées, Marseille, France1; Unité de Recherche pour les Maladies Infectieuses et Tropicales Emergentes, Unité Mixte de Recherche 6236, Marseille, France2; and Sanofi-Aventis Recherche et Développement, Sanofi-Aventis, Toulouse, France3

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The in vitro activity of ferroquine (FQ) (SR97193), a 4-aminoquinoline antimalarial compound that contains a ferrocenic nucleus, against 15 Plasmodium falciparum strains was assessed and compared with those of chloroquine (CQ), quinine (QN), monodesethylamodiaquine (MDAQ), and mefloquine (MQ). These 15 strains were genotyped for polymorphisms in quinoline resistance-associated genes such as PfCRT, PfMDR1, PfMRP, and PfNHE-1. FQ was highly active against CQ-resistant parasites or in parasites with reduced susceptibility to QN, MDAQ, or MQ. Encouragingly, we did not find a correlation between responses to FQ and those to other quinoline drugs. These results suggest that no cross-resistance exits between FQ and CQ or quinoline antimalarial drugs. Mutations in codons 74, 75, 76, 220, 271, 326, 356, and 371 of the PfCRT gene; codons 86, 184, 1034, 1042, and 1246 of the PfMDR1 gene; and codons 191 and 437 of the PfMRP gene were not significantly associated with P. falciparum susceptibility to FQ. Neither the number of ms4760 DNNND or DNNNDHHN repeats in PfNHE-1 nor the profile of ms4760 was significantly associated with the FQ in vitro response. These data suggest the FQ may not interact with transport proteins in quinoline-resistant parasites. The present results justify further clinical trials of FQ in multidrug resistance areas.

Two of the current options to reduce the morbidity and mortality of malaria are chemoprophylaxis and chemotherapy. During the past 20 years, many strains of Plasmodium falciparum have become resistant to chloroquine and other antimalarial drugs (24). This has prompted a search for an effective alternative antimalarial drug with minimal side effects. The emergence and spread of parasites that are resistant to antimalarial drugs has caused an urgent need for novel compounds to be discovered and developed.

An approach to remove aminoquinoline resistance in parasites is to modify the position and the chemical nature of the substituents or the length of the side chain on the quinoline nucleus of the aminoquinoline (12, 34). Recently, many different metals have been incorporated into antimalarial agents (29). Indeed, several organometallic compounds based on chloroquine with a ferrocene nucleus localized at different sites have been synthesized (5–8). This approach is currently being developed by J. Brocard and colleagues (URA-CNRS 402, Lille, France), who have synthesized ferroquine (FQ) {i.e., 7-chloro-4-[(2-N,N’-dimethylaminomethyl)ferrocenylethylamino]quinoline} (Fig. 1). FQ is currently under phase II clinical trial investigations.

Only six previous studies investigated the activity of ferroquine against Plasmodium falciparum strains isolated from infected patients (1, 2, 10, 21, 28, 30). The drug susceptibilities of Plasmodium falciparum strains vary among different locations, where isolates have different antimalarial resistance backgrounds. It seems that ferroquine activity is independent of chloroquine resistance in Pl. falciparum (21), and ferroquine antimalarial activity is not influenced by polymorphisms in the PfCRT gene (Plasmodium falciparum chloroquine resistance transporter), which encodes a protein located in the parasite digestive vacuole and is involved in drug transport and chloroquine resistance (10, 11).

The objective of this study was to determine whether genetic polymorphisms in genes associated with quinoline resistance modulate in vitro responses to ferroquine. We assessed polymorphisms in genes that are potentially associated with quinoline resistance: PfCRT, PfMDR1, PfMRP, and PfNHE-1 (P. falciparum sodium/hydrogen exchanger), and PfMRP (P. falciparum multidrug resistance protein). There is strong evidence that PfCRT is associated with chloroquine resistance (18, 32). PfMDR1 is involved in mefloquine resistance (15, 31). The evidence of the involvement of PfNHE-1 in resistance is compelling but weaker than those for PfCRT or PfMDR1. PfNHE-1, which encodes a proton transporter localized to the plasma membrane, may alter quinine activity (4, 17). The evidence for PfMRP being involved in resistance is still debatable. However, it seems that PfMRP is associated with decreases in chloroquine and quinine susceptibility (20, 26, 33).

MATERIALS AND METHODS

Plasmodium falciparum cultures. Fifteen monoclonal strains isolated from patients from a wide panel of countries (Brazil, Cambodia, Cameroon, Comoros,
In vitro cross-resistance was measured by the pairwise correlation of IC_{50} values of all 15 strains. Neither FQ and CQ

The IC_{50} value was determined by nonlinear regression analysis of log-based dose-response curves (RiaMSart; Packard).

**Nucleic acid extraction.** Total genomic DNA of each strain was isolated by using the E.Z.N.A. blood DNA kit (Omega Bio-Tek, GA) extraction method. RNA of each strain was purified by using the QiAamp Blood Mini kit (Qiagen, Germany).

**Pfcr single-nucleotide polymorphisms (SNPs).** A 1.250-nucleotide-length fragment of the Pfcr gene was amplified by reverse transcription-PCR using primers F1sense (5'-TAA TTT CCT ACA TAT AAC AAA TTC-3') and F1antisense (5'-TTA TGT TGT TGT AAT TAT TGA ATC GAC-3') and sequenced using primers F2sense (5'-TAG GTG GAT GTT GCT TGT GTA-3') and F2antisense (5'-TGG AGC GTT GTT AAT TCT CCT TC-3') (16). Amplifications were performed according to the manufacturer's instructions (Access reverse transcription-PCR system kit; Promega, WI). Sequencing was conducted using ABI Prism Big Dye Terminator v1.1 (Applied Biosystems, CA) cycle sequencing ready reaction kits.

**Pfmdr1 SNPs.** Pfmdr1 was amplified by PCR using primers 5'-TTA CAT TTT ATT TGA TTT GTT TTG-3' and 5'-CAT CCT TTT TAG CAT TAT CAT AAT GAA-3' to amplify coding regions 86 and 184 and 5'-AGC GGT TTA GTA AAT ATT GGT-3' and 5'-ATG GGT TCT TGA CTA ACT ATT G-3' to amplify codons 1034, 1042, and 1246. Amplifications were performed with the Titanium PCR kit (Clontech Ozyme, France) according to the manufacturer's instructions. The amplified fragments were sequenced as previously described.

**Pfmrp SNPs.** PCR amplification followed by sequencing was used to detect SNPs in Pfmrp at positions 191 and 437. The primers used for amplification and sequencing were pfmrp-501F (5'-TT ATT AAT GAT ATA AAT AAT ATT GAT GTA AAT GAA-3') and pfmrp-1409R (5'-GCC CTA AAT ATT GAT GTA AAG-3').

**Pfmdr1 microsatellite profile.** A sequence containing the msa4760 microsatellite described previously (17) was amplified using primers pfhne-3802F (5'-TT ATT GAT GAA TATA AAG-3') and pfhne-4322R (5'-TT TTT TTT TAC TTT AAC TAA AAG-3'). The amplified fragments were sequenced as previously described.

**Statistical analysis.** Assessment of standard antimalarial drug cross-resistance with FQ was estimated by determining the coefficient of correlation (r) and coefficient of determination (R^2). The Kruskal-Wallis test or the Mann-Whitney U test was used, when appropriate, to compare equalities of populations for each mutation.

**RESULTS**

Fifteen *P. falciparum* strains were tested for their in vitro susceptibilities to FQ, CQ, QN, MQ, and MDAQ. FQ had a considerably higher level of activity than did all quinolines tested. The IC_{50} value for FQ ranged from 1.8 to 13.4 nM, with a 5.3 nM mean (standard deviation, ±3.2 nM) (Table 1).

In vitro cross-resistance was measured by the pairwise correlation of IC_{50} values of all 15 strains. Neither FQ and CQ
TABLE 2. Correlation of in vitro responses of 15 strains of *Plasmodium falciparum* to FQ, CO, QN, MQ, and MDAQ

<table>
<thead>
<tr>
<th>Drug</th>
<th>Drug partner</th>
<th>r</th>
<th>r²</th>
<th>P  value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FQ</td>
<td>CO</td>
<td>−0.147</td>
<td>0.0216</td>
<td>0.600</td>
</tr>
<tr>
<td>FQ</td>
<td>QN</td>
<td>−0.066</td>
<td>0.0044</td>
<td>0.816</td>
</tr>
<tr>
<td>FQ</td>
<td>MQ</td>
<td>−0.011</td>
<td>0.0001</td>
<td>0.970</td>
</tr>
<tr>
<td>FQ</td>
<td>MDAQ</td>
<td>+0.086</td>
<td>0.0074</td>
<td>0.760</td>
</tr>
<tr>
<td>CO</td>
<td>MQ</td>
<td>+0.756</td>
<td>0.5715</td>
<td>0.001</td>
</tr>
<tr>
<td>CO</td>
<td>MDAQ</td>
<td>+0.806</td>
<td>0.6496</td>
<td>0.0003</td>
</tr>
<tr>
<td>QN</td>
<td>MQ</td>
<td>−0.421</td>
<td>0.1697</td>
<td>0.118</td>
</tr>
<tr>
<td>QN</td>
<td>MDAQ</td>
<td>+0.728</td>
<td>0.5300</td>
<td>0.002</td>
</tr>
<tr>
<td>MQ</td>
<td>MDAQ</td>
<td>−0.585</td>
<td>0.3422</td>
<td>0.022</td>
</tr>
</tbody>
</table>

(\(r^2 = 0.0216\)) nor FQ and the other quinolines tested were correlated (Table 2). On the contrary, QQ and QN, CO and MDAQ, QN and MQ, QN and MDAQ, and MQ and MDAQ were significantly correlated.

The following mutations were identified for at least one strain: PfCRT M74I, N75E, K76T, A220S, Q271 (E/V), N326S, I356T, and I371R; PfMRP H191Y and S437A; and PfMDR1 N86Y, Y184F, S1034C, N1042D, and D1246Y (Table 2). Six different ms4760 microsatellite profiles of *Pbhe-1* were observed (Fig. 2). The numbers of DNNND and DDHNHNH NNHN repeats on ms4760 ranged from 1 to 4 and 1 to 2, respectively (Table 3).

Polymorphisms in the PfCRT, PfMDR1, or PfPMP gene were not associated with *P. falciparum* susceptibility to FQ (\(P > 0.386\)). On the contrary, in vitro resistance to CQ and reduced susceptibility to QN and MDAQ were significantly associated with mutations in codons 74, 75, 76, 220, 271, 326, 356, and 371 in the PfCRT gene (0.005 < \(P < 0.05\)) and in codons 191 and 437 in the PfPMP gene (\(P < 0.007\)). Reduced susceptibility to MQ was significantly associated with mutations in codons 74, 75, 76, 220, 271, 326, and 371 in the PfCRT gene (0.017 < \(P < 0.05\)) and in codons 191 and 437 in the PfPMP gene (\(P = 0.05\)). In addition, in vitro resistance to CQ and reduced susceptibility to QN were significantly associated with mutations in codons 1034 and 1042 in the PfMDR1 gene (\(P = 0.014\)).

The number of ms4760 DNNND repeats in *Pbhe-1* was not significantly associated with the FQ response (\(P = 0.923\)), in opposition to those of CQ, QN, and MDAQ (\(P < 0.066\)).

Statistical analysis was performed for various profiles including ms4760-6 and ms4760-7, which were the most commonly observed profiles. No significant association between FQ or MQ IC₅₀ and *Pbhe-1* ms4760 profiles was established. On the contrary, a significant association was observed for the most frequent profiles (ms4760-6 and ms4760-7) for CO, QN, and MDAQ (0.021 < \(P < 0.049\)). Profile 6 was significantly associated with reduced susceptibility to CO, QN, and MDAQ, whereas Profile 7 was significantly associated with a high level of in vitro resistance to CO, QN, and MDAQ.

**DISCUSSION**

FQ, a CO derivative, is highly active against CO-resistant *P. falciparum* laboratory strains (13) and against *P. falciparum* strains isolated from infected patients (1, 2, 10, 21, 28, 30). FQ shows good antimalarial and toxicity profiles in rodent malaria models (8). FQ is therefore an interesting candidate for clinical development. FQ is even highly active against parasites with reduced susceptibility to QN, MDAQ, or MQ. FQ is more active than CO, QN, MDAQ, and even MQ. Encouragingly, we did not find a correlation between FQ and the other quinoline drugs, i.e., CO, QN, MDAQ, or MQ. These results suggest that no cross-resistance between FQ and CQ or quinoline antimalarial drugs, exits. These data are in accordance with previous studies, which showed weak coefficients of determination, between 0.096 and 0.127, for correlation between FQ and CQ (2, 21, 28). The potency of FQ against CO-, QN-, MDAQ-, or MQ-resistant *P. falciparum* strains and the absence of cross-resistance suggest that both drugs have different modes of action or mechanisms of resistance. CQ is believed to act by concentrating in the parasite digestive vacuole and preventing the crystallization of toxic heme in the hemozoin, leading to membrane damage and parasite death (14, 35). Like CQ, FQ forms complexes with hematin in solution and is an inhibitor of β-hematin formation (9). Nevertheless, the absence of cross-resistance between FQ and the other quinolines suggests that FQ may not work exactly as does chloroquine, react with heme and hemozoin differently than the other quinolines, or have a different molecular target.

IC₅₀ values for FQ were found to be unrelated to mutations occurring in transport protein genes involved in quinoline antimalarial drug resistance, such as PfCRT, PfMDR1, PfPMP, or *Pbhe-1*. The absence of association with FQ activity and polymorphisms in the PfCRT gene is consistent with previous results for Cambodian isolates (10). These data suggest that FQ may not be expelled by transport proteins in quinoline-resistant parasites, possibly as a result of the strong affinity of *P. falciparum* for the iron moiety of the molecule (25). In comparison to CQ, the presence of a ferrocene moiety with a different shape, volume, lipophilicity, effects on basicity, and electrostatic profile dramatically modifies the pharmacological behavior of the parent drug (9). Therefore, FQ appears to present reduced affinity for the transporters involved in the resistance to CQ and quinoline drugs. This may partially explain the high level of activity of FQ against multidrug-resistant *P. falciparum* parasites. This is consistent with results that indicate that the ability of mutant PfCRT to confer CQ resistance is precisely

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**FIG. 2.** Sequences of *Pbhe-1* microsatellite ms4760 detected among the 15 studied *P. falciparum* strains. Profiles 1 to 7 were previously described (16).
TABLE 3. Pf\textit{mdr1}, Pf\textit{mrp}, and Pf\textit{nhe-1} polymorphisms in \textit{Plasmodium falciparum} strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pf\textit{mdr1} codon:</th>
<th>Pf\textit{mrp}</th>
<th>Pf\textit{nhe-1} microsatellite</th>
<th>DDNHNDNHHH</th>
<th>No. of Pf\textit{mrp} microsatellite repeats</th>
<th>No. of Pf\textit{nhe-1} microsatellite repeats</th>
</tr>
</thead>
<tbody>
<tr>
<td>W2</td>
<td>D</td>
<td>H</td>
<td>S</td>
<td>3</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>D6</td>
<td>D</td>
<td>H</td>
<td>A</td>
<td>3</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>FCM29</td>
<td>D</td>
<td>H</td>
<td>A</td>
<td>3</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>HB3</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>3</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>IMT Bc</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>3</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>

a Polymorphism types are detailed in Fig. 2. Boldface type indicates point mutations.

configured for CQ (22). Resistance was rapidly lost following subtle structural modifications of the basic diethylamino side chain linked to the 4-aminooxime ring structure. Cross-resistance was clearly evident with analogs that varied by only a single CH\textsubscript{2} group and absent when two CH\textsubscript{2} groups were removed or six were added (22). In addition, \textit{Dictyostelium discoideum} transformants expressing the CQ resistance phenotype Pf\textit{crt} were not able to expel piperaquine, a bisquinoline analog of CQ (27). The absence of an interaction of FQ with Pf\textit{crt} suggests that the phenotypic response to FQ would not be modified by resistance reversers such as verapamil. Nevertheless, the effects of reversers on aminooxime analogs are still debated. Verapamil did not affect the relative piperaquine response in \textit{D. discoideum} transformants expressing the CQ resistance phenotype Pf\textit{crt} at concentrations that completely reverse CQ resistance (27), while desipramine could reverse resistance to bisquinoline WR268,668 (3). The ability of verapamil to enhance the activity of a drug is inversely related to the log\textsubscript{10} of the drug (19). In addition, no resistance of \textit{P. falciparum} to FQ has been found in vitro in either patient isolates or laboratory-adapted strains under drug pressure (11).

In conclusion, FQ is highly active against parasites with reduced susceptibility to ON, MDAQ, or MQ. No cross-resistance between FQ and CQ, or quinoline antimalarial drugs, exists. IC\textsubscript{50} values for FQ were found to be unrelated to mutations occurring in transport protein genes involved in quinoline antimalarial drug resistance, such as Pf\textit{crt}, Pf\textit{mdr1}, Pf\textit{mrp}, and Pf\textit{nhe-1}. The present results justify clinical trials of FQ in multidrug resistance areas. A phase II study is now in progress in Gabon.

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