Ferroocene-chloroquine analogues as antimalarial agents: *in vitro* activity of ferrochloroquine against 103 Gabonese isolates of *Plasmodium falciparum*


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The *in vitro* activities of ferrochloroquine, chloroquine, quinine, mefloquine, halofantrine, amodiaquine, primaquine, atovaquone and artesunate were evaluated against *Plasmodium falciparum* isolates from children with uncomplicated malaria from Libreville (Gabon), using an isotopic, micro, drug susceptibility test. The IC₅₀ values for ferrochloroquine were in the range 0.43–30.9 nM and the geometric mean IC₅₀ for the 103 isolates was 10.8 nM (95% CI 8.6–13.5 nM), while the geometric means for chloroquine, quinine, mefloquine, amodiaquine and primaquine were 370 nM, 341 nM, 8.3 nM, 18.1 nM and 7.6 μM, respectively. Ferrochloroquine was active against *P. falciparum* isolates, 95% of which showed *in vitro* resistance to chloroquine. Weak positive significant correlations were observed between the responses to ferrochloroquine and that to chloroquine, amodiaquine and quinine, but too low to suggest cross-resistance. There was no significant correlation between the response to ferrochloroquine and those to mefloquine, halofantrine, primaquine, atovaquone or artesunate. Ferrochloroquine may be an important alternative drug for the treatment of chloroquine-resistant malaria.

Introduction

In the absence of effective and practical preventive measures, the only current options for reducing the morbidity and mortality of malaria are chemoprophylaxis and chemotherapy. Therefore the increasing prevalence of strains of *Plasmodium falciparum* resistant to chloroquine and other antimalarial drugs is a serious problem for malaria control. Failures of antimalarial prophylaxis with chloroquine, the combination of chloroquine and proguanil, mefloquine, and treatment failures with halofantrine and quinine have been observed in Africa. This emergence and spread of parasite resistance to currently used antimalarial drugs means that new compounds with minimal side effects need to be discovered and developed by identification of novel chemotherapeutic targets.

Iron is an essential element for the growth of all living organisms. Furthermore, iron has a critical role in host–parasite interactions. Two approaches based on iron could be proposed for drug design and therapy. First, iron chelation therapy, which was once considered a suitable treatment for various infectious diseases, including malaria. The second approach is to use the avidity of *Plasmodium* for free iron. An effective way of removing the chloroquine resistance of parasites could be by the addition of iron to a chloroquine molecule. Organometallic compounds based on chloroquine with a ferrocene nucleus localized at different sites were synthesized. These organometallic chloroquine analogues showed high *in vitro* activities against clones and strains of *P. falciparum* and *in vivo* on mice infected with *Plasmodium berghei* N. and *Plasmodium yoelii* NS. One such analogue is the ferrochloroquine...
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**Drugs**

Ferrochloroquine was obtained from Laboratoires Pierre Fabre, chloroquine diphosphate, quinine hydrochloride, amodiaquine and primaquine from Sigma Chemicals (St Louis, MO, USA), mefloquine from Roche (Neuilly sur Seine, France), artesunate from Sanofi Winthrop (Gentilly, France) and atovaquone from Glaxo Wellcome (Hertfordshire, UK). Stock solutions were prepared in sterile distilled water for chloroquine diphosphate, amodiaquine and primaquine, and in methanol for quinine, halofantrine, mefloquine, atovaquone and artesunate. Two-fold serial dilutions were prepared in sterile distilled water for all these antimalarial drugs. Final concentrations ranging from 25 to 3200 nM chloroquine, 50 to 3200 nM quinine, 3.1 to 400 nM amodiaquine, 0.25 to 32 nM halofantrine, 3.12 to 400 nM mefloquine, 0.8 to 100 nM atovaquone, 0.4 to 100 nM artesunate and 0.025 to 100 μM primaquine were distributed in triplicate into Falcon 96-well flat-bottomed plates (Becton Dickinson, Franklin Lakes, NJ, USA), which were dried. Stock solution and two-fold serial dilutions of ferrochloroquine in RPMI 1640 ranging from 0.5 to 1502 nM were prepared just before use and distributed in triplicate into Falcon 96-well flat-bottomed plates before *in vitro* assay.

**Materials and methods**

**Isolates of *P. falciparum***

Between June and December 1999, 271 fresh *P. falciparum* isolates were obtained from children with uncomplicated malaria from Libreville (Gabon). Venous blood was collected before treatment in Vacutainer ACD tubes (Becton Dickinson, Rutherford, NJ, USA) and transported at 4°C to our laboratory in Marseille. Informed oral consent was obtained from parents before collection of blood. The study protocols were reviewed and approved by the Directeur of Centre Hospitalier de Libreville (CHL). Thin blood smears were stained using a RAL kit (Réactifs RAL, Paris, France) and washed using a cell harvester (FilterMate Cell Harvester, Packard). Filter microplates were dried and 25 μL of scintillation cocktail (Micoscent O, Packard) was placed in each well. Radioactivity incorporated by the parasites was measured using a scintillation counter (Top Count, Packard).

Ferrochloroquine cross-resistance with the other antimalarials (chloroquine, quinine, amodiaquine, mefloquine, halofantrine, primaquine, atovaquone and artesunate) was estimated by Pearson correlation coefficient (r) and coefficient of determination (r²).
**Ferrocene-chloroquine analogue antimalarials**

**Results**

The volume of blood of some samples did not allow testing of all the drugs. The following proportions of isolates were successfully cultured for each drug tested: 103 of 141 for ferrochloroquine, 102 of 141 for chloroquine, 98 of 122 for halofantrine, 88 of 122 for mefloquine, 71 of 86 for amodiaquine and primaquine, 67 of 86 for atovaquone and 65 of 86 for quinine and artesunate. Average parameter estimates for the nine compounds against all isolates are given in Table I.

The IC_{50} values for ferrochloroquine were in the range 0.43–30.9 nM and the geometric mean IC_{50} for the 103 isolates was 10.8 nM (95% CI 8.6–13.5 nM).

Weak positive significant correlations were observed between the responses to ferrochloroquine and chloroquine, ferrochloroquine and amodiaquine, and ferrochloroquine and quinine. There was no significant correlation between the response to ferrochloroquine and that to mefloquine, halofantrine, primaquine, atovaquone and artesunate (Table II).

**Discussion**

The results of the present study show the high activity of ferrochloroquine against Gabonese isolates of *P. falciparum*. Ferrochloroquine was more active than chloroquine (34-fold greater), quinine, amodiaquine and primaquine. Halofantrine, atovaquone and artesunate were more active. Ferrochloroquine was highly active against isolates from an area where 95% of the isolates were resistant in vitro to chloroquine, 19% resistant to atovaquone and 11% resistant to quinine and to artesunate.

A positive correlation between the IC_{50} of two antimalarial drugs may suggest in vitro cross-resistance, but the relationship between in vitro and in vivo resistance depends on the level of resistance and coefficients of corre-

### Table I. *In vitro* activities of the nine drugs tested against Gabonese fresh isolates of *P. falciparum*

<table>
<thead>
<tr>
<th>Drug</th>
<th>No. of isolates</th>
<th>Mean IC_{50} (nM)^a</th>
<th>95% CI</th>
<th>Resistant isolates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrochloroquine</td>
<td>103</td>
<td>10.8</td>
<td>8.6–13.5</td>
<td>ND</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>102</td>
<td>370</td>
<td>319–429</td>
<td>95</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>88</td>
<td>8.3</td>
<td>7.2–9.6</td>
<td>1</td>
</tr>
<tr>
<td>Halofantrine</td>
<td>98</td>
<td>0.8</td>
<td>0.7–1.0</td>
<td>2</td>
</tr>
<tr>
<td>Quinine</td>
<td>65</td>
<td>341</td>
<td>293–398</td>
<td>11</td>
</tr>
<tr>
<td>Amodiaquine</td>
<td>71</td>
<td>18.1</td>
<td>16.1–20.1</td>
<td>0</td>
</tr>
<tr>
<td>Primaquine</td>
<td>71</td>
<td>7600</td>
<td>6600–8700</td>
<td>ND</td>
</tr>
<tr>
<td>Atovaquone</td>
<td>67</td>
<td>3.3</td>
<td>2.7–3.9</td>
<td>19</td>
</tr>
<tr>
<td>Artesunate</td>
<td>65</td>
<td>2.9</td>
<td>2.3–3.7</td>
<td>11</td>
</tr>
</tbody>
</table>

Cut-off values for *in vitro* resistance to chloroquine, mefloquine, halofantrine, quinine, amodiaquine, atovaquone and artesunate are 100, 30, 6, 800, 80, 6 and 10.5 nM, respectively.

ND, not determined, cut-off value unknown.

^aValues are the geometric mean IC_{50}.

### Table II. Correlation of *in vitro* responses of Gabonese isolates of *P. falciparum* to ferrochloroquine, chloroquine, mefloquine, halofantrine, quinine, amodiaquine, primaquine, atovaquone and artesunate

<table>
<thead>
<tr>
<th>Drug pair</th>
<th>No.</th>
<th>r</th>
<th>r^2</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrochloroquine</td>
<td>102</td>
<td>0.356</td>
<td>0.127</td>
<td>0.0002</td>
</tr>
<tr>
<td>Ferrochloroquine</td>
<td>65</td>
<td>0.319</td>
<td>0.102</td>
<td>0.0095</td>
</tr>
<tr>
<td>Ferrochloroquine</td>
<td>88</td>
<td>-0.028</td>
<td>0.001</td>
<td>0.7971</td>
</tr>
<tr>
<td>Ferrochloroquine</td>
<td>98</td>
<td>-0.016</td>
<td>&lt;0.001</td>
<td>0.8729</td>
</tr>
<tr>
<td>Ferrochloroquine</td>
<td>71</td>
<td>0.354</td>
<td>0.125</td>
<td>0.0025</td>
</tr>
<tr>
<td>Ferrochloroquine</td>
<td>71</td>
<td>0.127</td>
<td>0.016</td>
<td>0.2911</td>
</tr>
<tr>
<td>Ferrochloroquine</td>
<td>67</td>
<td>0.106</td>
<td>0.011</td>
<td>0.3940</td>
</tr>
<tr>
<td>Ferrochloroquine</td>
<td>65</td>
<td>0.170</td>
<td>0.029</td>
<td>0.1746</td>
</tr>
</tbody>
</table>

r = Pearson correlation coefficient, and r^2 = coefficient of determination.
lation ($r$) and determination ($r^2$). We noted weak positive significant correlations ($r^2 < 0.15$) between the responses to ferrochloroquine and chloroquine, ferrochloroquine and amodiaquine, and ferrochloroquine and quinine. Only 10% of the variation of response to ferrochloroquine is explained by response variation to quinine, 12.5% for amodiaquine and 13% for chloroquine. From these data, we consider that no cross-resistance exists between ferrochloroquine and chloroquine, quinine and amodiaquine. We can only suggest that common features in drug uptake and/or mode of action or resistance may explain these weak positive correlations.

There is still uncertainty about the mode of action of chloroquine and the mechanism of resistance to it. It has been hypothesized that, as weak bases, chloroquine and its close analogues follow the pH gradient and accumulate in the food vacuole of the susceptible parasites.18 The most convincing explanation of its activity lies in its capacity to interfere with haemoglobin degradation in the food vacuole by raising the vacuolar pH,19 and/or by inhibition of the polymerization of the free haem by the formation of toxic haem–chloroquine complex.20 It was initially believed that this reaction was catalysed by a haem polymerase enzyme that was inhibited by chloroquine.21 However, haematin polymerization is a physical process and is not enzyme mediated.22 In view of their closely related structures, the modes of action of this ferrocene analogue and chloroquine could be identical. It has been demonstrated that this ferrocene molecule needs to be bound covalently to the chloroquine to inhibit the resistance of the parasites and that the ferrocene by itself did not have antimalarial activity but enhanced the effectiveness of the chloroquine when it was enclosed inside the molecule.17

Resistant parasites actively expel chloroquine,23 probably by means of a transporter [P-glycoprotein homologue (Pgh) 1] coded by a multidrug-resistant (mdr) gene.24 Therefore, it is likely that the mode of action of this gene is caused by mutations in a number of genes.25 Su and others26 have produced evidence from linkage analysis of a P. falciparum cross that a gene encoding a unique, c. 330 kDa protein with complex polymorphisms located on chromosome 7 determines chloroquine resistance. Accumulation of amodiaquine was correlated with accumulation of chloroquine and accumulation of both drugs was significantly reduced in chloroquine-resistant isolates.27 Previous studies have established an association between in vitro chloroquine resistance and the presence of tyrosine at the position encoded by codon 86 of the pfmdr1 gene of P. falciparum in a range of sub-Saharan isolates.28 The contribution of this polymorphism to amodiaquine resistance has been also assessed,29 and may explain cross-resistance between amodiaquine, chloroquine and ferrochloroquine. A similar effectiveness of the synergic combination of ferrochloroquine and calcium inhibitors like verapamil has been observed.17 The ferrochloroquine could inhibit the efflux of chloroquine because of the permease having a lower affinity for the modified chloroquine. However, ferrocene probably has a mechanism of action different from that of verapamil on the mode of resistance of the parasites.

Dorn and others30 have demonstrated a correlation for chloroquine, amodiaquine, quinine, mefloquine and halofantrine between the inhibition of haematin polymerization in vitro and the inhibition of Plasmodium falciparum growth in culture, which confirms haematin polymerization as the likely target of quinoline blood schizonticides. However, we noted only positive significant correlations between the responses to ferrochloroquine and chloroquine, and ferrochloroquine and amodiaquine but not between the responses to ferrochloroquine and quinine, mefloquine or halofantrine. The explanation of the inhibition of haematin polymerization as the mode of action of ferrochloroquine is not completely satisfactory.

The lack of correlation between ferrochloroquine and primaquine, atovaquone or artesunate is likely to be owing to the difference in the targets of the drugs. Atovaquone, a hydroxynaphthoquinone, inhibits the plasmodium mitochondrial respiratory process by blocking electron transfer mechanisms at the cytochrome bcl complex,31 indirectly inhibiting dihydroorotate dehydrogenase,32 the enzyme catalysing the fourth step of the pathway for de novo biosynthesis of pyrimidine nucleotides and inducing decreases in the level of dNTPs (deoxynucleoside 5’ triphosphates).33 Artesunate, like all the artemisinin compounds, contains stable endoperoxide bridges. One of the major artemisinin target proteins in P. falciparum is the translationally controlled tumour protein (TCTP) homologue, which appears to bind haem and to react selectively with artemisinin.34 Primaquine demonstrates schizontocidal, gametocytocidal and sporonticidal activities.35,36 Its mode of action is not clearly defined.

Organometallic chloroquine analogues showed high in vivo activities in mice infected with P. berghei N. and P. yoelii NS.14 Toxicological tests are necessary to show a low incidence and severity of adverse effects for prophylactic use of these compounds. No significant evidence of toxicity of ferroenes was detectable following oral administration in dogs.37 Ferrochloroquine may be an important alternative drug for the treatment of chloroquine-resistant malaria.

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