IN VITRO SUSCEPTIBILITY OF AFRICAN ISOLATES OF PLASMODIUM FALCIPARUM FROM GABON TO PYRONARIDINE


Unité de Parasitologie, Institut de Médecine Tropicale du Service de Santé des Armées, Le Pharo, Marseille, France; Département de Parasitologie, Mycologie et Médecine Tropicale, Faculté de Médecine et des Sciences de la Santé, Libreville, Gabon; Service Médical, 6ème Bataillon d’Infanterie de Marine, Libreville, Gabon

Abstract. The in vitro activity of pyronaridine was evaluated against 62 isolates of Plasmodium falciparum from Libreville, Gabon using an isotopic, drug susceptibility microtest and was compared with amodiaquine, chloroquine, quinine, and halofantrine activities. The mean 50% inhibitory concentration (IC₅₀) values of the 62 isolates from Gabon to pyronaridine was 3.0 nM (95% confidence interval [CI] = 2.1–3.9). Pyronaridine was less potent against chloroquine-resistant isolates than chloroquine-susceptible isolates but more potent than chloroquine against chloroquine-resistant parasites. The cut-off value for in vitro reduced susceptibility to pyronaridine was an IC₅₀ > 15 nM. Two isolates (3%) showed an IC₅₀ > 15 nM. A significant positive correlation was found between the activities of pyronaridine and chloroquine (r² = 0.26, P < 0.001), pyronaridine and quinine (r² = 0.36, P < 0.001), pyronaridine and amodiaquine (r² = 0.55, P < 0.001), and pyronaridine and halofantrine (r² = 0.50, P < 0.001). This correlation suggests in vitro cross-resistance or at least in vitro cross-susceptibility, which is not necessarily predictive of cross-resistance in vivo. The present in vitro findings require comparison with those of clinical studies.

The only current options for reducing the morbidity and mortality of malaria, especially in Africa, are chemoprophylaxis and chemotherapy. Therefore, the increasing prevalence of strains of Plasmodium falciparum resistant to chloroquine and other antimalarial drugs poses a serious problem for control of malaria. Failures of antimalarial prophylaxis with chloroquine, a combination of chloroquine and proguanil, and mefloquine and clinical failures with halofantrine and quinine have been observed in Africa. There is an urgent need to find and develop alternative drugs against chloroquine-resistant P. falciparum.

One group of alternative antimalarial drugs is Mannich bases, particularly pyronaridine. This drug was synthesized in China at the end of 1970. It is a blood schizonticidal drug that is highly effective against multidrug-resistant P. falciparum laboratory strains and clones. Clinical trials of pyronaridine involving more than 1,000 Chinese patients have shown its high efficacy against P. falciparum and P. vivax with few side effects. Pyronaridine was also shown to be more effective than chloroquine in Cameroon. In vivo activities of Mannich base antimalarials, including pyronaridine, have been explored against rodent malaria parasites and against simian malaria.

However, clinical and field evaluation of pyronaridine is not yet complete. Few in vitro studies have been performed on field isolates. The few previous works included either clones or strains or a restricted number of strains or isolates. The aim of this study was to determine the in vitro activity of pyronaridine on 62 P. falciparum isolates, and compare this with that of chloroquine, quinine, halofantrine, and amodiaquine. Such studies will also help determine the appropriate threshold values for parasite resistance to pyronaridine.

MATERIALS AND METHODS

Isolates of P. falciparum. This study was approved by the Gabonese Ethics Committee of the Faculty of Medicine of Libreville. Between April and July 1997, we analyzed the drug sensitivity patterns of 62 fresh P. falciparum isolates obtained from hospitalized children (six months to 15 years old) in Libreville, Gabon with uncomplicated or severe malaria. Informal oral consent was obtained from the parents of the children before the collection of blood samples. Venous blood was collected into Vacutainer® ACD tubes (Becton Dickinson, Rutherford, NJ) before treatment and transported at 4°C to our laboratory in Marseille within 96 hr. Thin blood smears were stained using an RAL® kit (Réactifs RAL, Paris, France) and examined to determine parasite density and confirm monoinfection by P. falciparum. Samples with parasitemias ranging from 0.03% to 7.8% were used to test drug sensitivity. Parasitized erythrocytes were washed three times in RPMI 1640 medium (Gibco-BRL, Paisley, United Kingdom). If parasitemia exceeded 0.8%, infected erythrocytes were diluted to 0.5–0.8% with uninfected erythrocytes and resuspended in culture medium to a hematocrit of 1.5%. Susceptibility to pyronaridine, amodiaquine, chloroquine, quinine, and halofantrine was determined after suspension in RPMI 1640 medium supplemented with 10% human serum (pooled from different A+ or AB, non-immune individuals from the area of malaria endemicity) and buffered with 25 mM HEPES and 25 mM NaHCO₃.

Drugs. Pyronaridine phosphate was obtained from the World Health Organization (WHO) (batch no. 210642), chloroquine diphosphate, quinine hydrochloride, and amodiaquine dihydrochloride from Sigma (St. Louis, MO), and halofantrine hydrochloride from Smith Kline & French (Paris, France). Stock solutions were prepared in sterile distilled water for pyronaridine phosphate, chloroquine diphosphate, and amodiaquine dihydrochloride and in methanol for quinine and halofantrine (previous studies in our laboratory showed that methanol had no cytotoxicity on parasite growth and that there was no precipitation of antimalarials when dilutions were made in water). Two-fold serial dilutions were prepared in sterile distilled water. Final concentrations, which ranged from 0.8 to 100 nM for pyronaridine, 25–3,200 nM for chloroquine, 50–3,200 nM for quinine, 3.1–400 nM for amodiaquine, and 0.25–32 nM for halofantrine.
were distributed in triplicate into Falcon 96-well, flat-bottom plates (Becton Dickinson Labware, Franklin Lakes, NJ).

The chloroquine-susceptible D6  \( P. falciparum \) clone (Sierra Leone) and the chloroquine-resistant W2 clone (Indochina) were used as references to test each batch of plates. Reference clones were maintained in continuous culture and synchronized twice with sorbitol.

In vitro assay. For in vitro isotopic microtests, suspensions of parasitized erythrocytes (200 µl/well) were distributed in 96-well plates preseeded with antimalarial agents. Parasite growth was assessed by adding 1 µCi of \(^3\)H-hypoxanthine with a specific activity 14.1 Ci/mmol (New England Nuclear Products, Dreieich, Germany) to each well. Plates were incubated for 42 hr at 37°C in an atmosphere of 10% \( O_2 \), 6% \( CO_2 \), and 84% \( N_2 \), and a humidity of 95% (optimum conditions in our laboratory). Immediately after incubation the plates were frozen then thawed to lyse erythrocytes. The contents of each well were collected on standard filter microplates (Unifilter\textsuperscript{\textregistered} GF/B; Packard Instrument Company, Meriden, CT) and washed using a cell harvester (FilterMate\textsuperscript{\textregistered} Cell Harvester; Packard Instrument Company). Filter microplates were dried and 25 µl of scintillation cocktail (Microscint\textsuperscript{\textregistered} O; Packard Instrument Company) was placed in each well. Radioactivity incorporated by the parasites was measured using a scintillation counter (TopCount\textsuperscript{\textregistered}; Packard Instrument Company).

The 50% inhibitory concentration (IC\textsubscript{50}), i.e., the drug concentration corresponding to 50% of the uptake of \(^3\)H-hypoxanthine by the parasites in drug-free control wells, was determined by nonlinear regression analysis of log-dose/response curves. Data were expressed as the geometric mean IC\textsubscript{50} and 95% confidence intervals (95% CIs) were calculated. The unpaired \( t \)-test was used to compare IC\textsubscript{50} values from chloroquine-susceptible and chloroquine-resistant isolates.

Assessment of standard antimalarial cross-resistance with pyronaridine was estimated by Pearson correlation coefficient (\( r \)) and coefficient of determination (\( r^2 \)).\textsuperscript{19} A positive correlation in the response to two drugs may be interpreted as resistance to the first drug facilitating the resistance to the other drug.\textsuperscript{20} Isolates were considered as chloroquine-resistant if the IC\textsubscript{50} was greater than 100 nM. Cut-off values for resistance to amodiaquine, quinine, and halofantrine were 80 nM, 500 nM, and 6 nM, respectively. The in vitro threshold value to antimalarials has been defined statistically (> 2 SD above the mean). Only in vitro resistance to chloroquine evaluated statistically had been confirmed by correlation with in vivo therapeutic effectiveness.\textsuperscript{21} We have determined statistically (mean IC\textsubscript{50} + 2 SD) the cut-off value for pyronaridine-reduced susceptibility as 15 nM in a previous study in Senegal.\textsuperscript{22}

### RESULTS

The following proportions of isolates were successfully cultured for each drug tested: 59 of 62 for chloroquine, 61 of 62 for halofantrine, and 62 of 62 for pyronaridine, amodiaquine, and quinine. The IC\textsubscript{50} values for pyronaridine ranged from 0.8 to 17.9 nM (mean IC\textsubscript{50} = 3.0 nM and 95% CI = 2.1–3.9 nM).

Based on our criterion (IC\textsubscript{50} > 100 nM), 51 of 59 fresh isolates of  \( P. falciparum \) studied were considered as chloroquine-resistant (mean IC\textsubscript{50} = 238 nM, 95% CI = 191–285 nM). Mean IC\textsubscript{50} values for chloroquine-resistant and chloroquine-susceptible isolates were 291 nM and 56 nM, respectively, (ratio of 5.2). As shown in Table 1, pyronaridine had high antimalarial activity against both the chloroquine-susceptible and chloroquine-resistant isolates with mean IC\textsubscript{50} values of 1.5 nM and 3.3 nM, respectively. Pyronaridine was less potent against chloroquine-resistant isolates. Nevertheless, the ratios of mean IC\textsubscript{50} chloroquine/pyronaridine values for the chloroquine-susceptible and chloroquine-resistant parasites were 37 and 88, respectively. These results indicate that although pyronaridine was less potent against chloroquine-resistant isolates, the activity difference is less than that for chloroquine.

Based on previous data (mean IC\textsubscript{90} + 2 SD), the cut-off value for in vitro reduced susceptibility to pyronaridine is an IC\textsubscript{90} greater than 15 nM.\textsuperscript{22} Only two isolates (3%) showed an IC\textsubscript{90} > 15 nM.

There was a significant positive correlation between responses to pyronaridine, chloroquine, quinine, halofantrine, and amodiaquine, suggesting in vitro cross-resistance or, at least in vitro cross-susceptibility (Table 2).

### DISCUSSION

We observed in vitro chloroquine-resistance (86%) and reduced susceptibilities to cycloguanil (71%) and to chloroquine plus cycloguanil (67%) (Pradines B, unpublished data). The present study confirms the high level of in vitro antimalarial activity of pyronaridine reported by others workers using field isolates or culture-adapted strains of  \( P. falciparum \).

### Table 1

<table>
<thead>
<tr>
<th>Drug</th>
<th>Chloroquine-susceptible isolates (n = 8)</th>
<th>Chloroquine-resistant isolates (n = 51)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC\textsubscript{50} (nM)</td>
<td>95% confidence limits</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>56</td>
<td>36–75</td>
</tr>
<tr>
<td>Pyronaridine</td>
<td>1.5</td>
<td>1.0–1.9</td>
</tr>
</tbody>
</table>

* Values are the geometric mean 50% inhibitory concentrations (IC\textsubscript{50}). Threshold IC\textsubscript{50} value for resistance to chloroquine is >100 nM.

### Table 2

<table>
<thead>
<tr>
<th>Drug pair</th>
<th>r</th>
<th>r²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyronaridine</td>
<td>0.74</td>
<td>0.55</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pyronaridine</td>
<td>0.71</td>
<td>0.50</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pyronaridine</td>
<td>0.60</td>
<td>0.36</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>0.51</td>
<td>0.26</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Quinine</td>
<td>0.66</td>
<td>0.44</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Halofantrine</td>
<td>0.60</td>
<td>0.36</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Amodiaquine</td>
<td>0.46</td>
<td>0.21</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Quinine</td>
<td>0.74</td>
<td>0.55</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Amodiaquine</td>
<td>0.61</td>
<td>0.37</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Halofantrine\textsuperscript{†}</td>
<td>0.67</td>
<td>0.45</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* r = Pearson correlation coefficient; r² = coefficient of determination.
† n = 61.
‡ n = 59.
falciparum from Thailand (mean IC₅₀ = 10 nM) using the WHO in vitro microtest technique⁶⁰ or from Central and West Africa using isotopic semi-microtest (mean IC₅₀ = 8 nM).⁷⁷ Nevertheless, our mean IC₅₀ value of 3 nM was lower. Only a few (3%) isolates with decreased in vitro susceptibility to pyronaridine (IC₅₀ > 15 nM) were observed. Similar results were reported in Senegal.²²

However, there are conflicting reports on the correlation of P. falciparum responses to pyronaridine and chloroquine. Pyronaridine appeared to be equally effective in vitro against 37 isolates from two areas of Thailand with different chloroquine-resistance levels.¹⁰ Similarly, Basco and Le Bras showed no correlation between resistance to pyronaridine and chloroquine for 31 isolates from Central and West Africa.¹⁷ In the present study, the positive correlation between chloroquine and pyronaridine sensitivities (r = 0.51, P < 0.001) confirmed previous work on isolates from Thailand,¹⁸ from Somalia,²⁶ and Senegal.²² Twenty-six percent of the pyronaridine response variations were explained by the variation in the chloroquine responses. This significant positive correlation may suggest in vitro cross-resistance or at least in vitro cross-susceptibility, which is not necessarily predictive of cross-resistance in vivo. Nevertheless, cross-resistance with existing antimalarials is a potential factor in the early appearance of resistance to novel compounds. Since the mechanisms underlying the antimalarial action and resistance of chloroquine and pyronaridine are still unknown, we can only speculate about a positive correlation.

It has been hypothesized that as a weak base, chloroquine and its close analogs follow the pH gradient and accumulate in the food vacuole of susceptible parasites.²¹ Chloroquine may interfere with hemoglobin degradation in the food vacuole by raising the vacuolar pH.²⁴ It was postulated that chloroquine and quinine inhibited hematin polymerization in the parasite. It was initially believed that this reaction was catalyzed by heme polymerase enzyme that was inhibited by the role of quinoline blood schizonticides in the inhibition of hematin polymerization.³² However, other studies showed that the acquisition of reduced halofantrine susceptibility was accompanied by similar shifts in sensitivity to quinine, mefloquine, and other antimalarial agents containing the methanolic function, but increased susceptibility to chloroquine. This suggests that the methanolic functional group is essential for activity or is of importance in the resistance mechanism,³³ resistance to halofantrine, quinine, and mefloquine is related to reduced drug accumulation within the parasite, and that this can be achieved without overexpression of Pgh 1.³⁴ Pyronaridine contains a methanolic functional group.

Despite the positive correlations of 62 P. falciparum isolates for resistance to pyronaridine, chloroquine, quinine, halofantrine, and amodiaquine, which are not necessarily predictive of cross-resistance in vivo, high activity of pyronaridine is evident, even against chloroquine-resistant P. falciparum. Pyronaridine may be an important alternative drug for the treatment of chloroquine-resistant malaria. In addition, pyronaridine is well-tolerated, and its side effects are mild.³⁵ However, in vitro cross-resistance reinforces the idea that novel antimalarials should not be deployed for monotherapy. There is an urgent need to find a rational partner compound with which pyronaridine can be administered to prolong its potential usefulness before resistance begins to emerge. Potentiation between pyronaridine and artemether was observed against simian parasites.³⁶ If Mannich bases are used for treatment of malaria, it will be important to continuously monitor the response of parasites in different geographic regions.

Acknowledgments: We thank Dr. V. Thulliez (Paediatriques Department, Libreville Hospital) for recruitment of children, M. Fortunée for technical assistance and availability for the field work, D. Fouard for help in transporting isolates, and the staff of Institut de Medecine Tropicale du Service de Sante des Armees (P. Bigot, R. Ges, J. Mosnier, D. Ragot, and Y. Trullemans) for technical support.

Financial support: This work was supported by la Direction Centrale du Service de Sante des Armées and the Groupe de Recherche en Parasitologie.

REFERENCES