In-vitro activity of pyronaridine and amodiaquine against African isolates (Senegal) of *Plasmodium falciparum* in comparison with standard antimalarial agents


The in-vitro activities of pyronaridine, amodiaquine, chloroquine and quinine were evaluated against 161 isolates of *Plasmodium falciparum* from Senegal (Dielmo, Ndiop and Pikine), using an isotopic, micro, drug susceptibility test. The mean IC_{50} values (50% inhibitory concentration) for pyronaridine and amodiaquine were 3.8 nM (95% confidence interval (95% CI), 3.1–4.4) and 12.0 nM (95% CI, 10.0–14.0 nM), respectively. Pyronaridine and amodiaquine were more active than chloroquine against susceptible parasites. However, both drugs were significantly less active (*P* < 0.002 and *P* < 0.025) against chloroquine-resistant isolates than against chloroquine-susceptible isolates. Based on statistical calculation using the present data (mean IC_{50} + 2 S.D.), the cut-off value for in-vitro susceptibility to pyronaridine is IC_{50} < 15 nM; for eight isolates (5%) the IC_{50} was >15 nM. No isolates tested showed resistance to amodiaquine (IC_{50} > 80 nM). Significant positive correlations, suggesting cross-resistance among these drugs *in vitro*, were found between pyronaridine and chloroquine (*r^2* = 0.19, *P* < 0.001), pyronaridine and quinine (*r^2* = 0.44, *P* < 0.001), pyronaridine and amodiaquine (*r^2* = 0.34, *P* < 0.001), amodiaquine and chloroquine (*r^2* = 0.14, *P* < 0.001), and amodiaquine and quinine (*r^2* = 0.21, *P* < 0.001). The present in-vitro findings require comparison with clinical studies.

**Introduction**

In the absence of effective and practical preventive measures, 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, with combination of chloroquine and proguanil, with 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 are the Mannich bases, especially pyronaridine and amodiaquine. Pyronaridine, a substituted 1-aza-acridine with a ring system similar to that of mepacrine and also a substituted 1,5-naphthyridine, was synthesized in China in 1970. Its side-chain is structurally similar to that of two quinoline-type Mannich bases: amodiaquine and, to an even greater extent, amopyroquine. Clinical trials of pyronaridine, involving more than 1000 Chinese patients, have shown its high efficacy against *P. falciparum* and *Plasmodium vivax*, with few side effects. Pyronaridine was also shown to be more effective than chloroquine in Cameroon. The in-vivo activities of Mannich base antimalarials, including pyronaridine, have been explored against rodent malaria parasites and against simian malaria. Pyronaridine is a blood schizonticidal drug which is highly effective against multidrug-resistant *P. falciparum* laboratory strains and clones. Recently, there has been renewed interest in amodiaquine. A modiomyquine was more effective than chloroquine, with lower parasitological and clinical failure rates in the treatment of infected Gambian children. Because of bone marrow and liver toxicity, amodiaquine can no longer be recommended for prophylaxis. However, no serious toxicity has been reported for treatment of acute malaria. The clinical and field evaluation of pyronaridine and
amodiaquine is not yet complete. The aims of this study were to assess the in-vitro activities of pyronaridine and amodiaquine against 161 isolates of \emph{P. falciparum}, and to compare their activities with those of chloroquine and quinine. Such field studies will also help determine the appropriate threshold values for parasite resistance to pyronaridine, which are currently unknown. Previous workers included either clones, or a restricted number of strains or isolates.

\textbf{Materials and methods}

\textbf{Isolates of} \emph{P. falciparum}

Between October and December 1996, 161 fresh isolates of \emph{P. falciparum} were prepared from samples obtained in Dielmo, Ndiop (280 km south-east of Dakar) in the Fatick region of Senegal and in Pikine. Patients from Pikine were recruited at the public centre, while patients from Dielmo and Ndiop were recruited at home by daily active case detection during a longitudinal study of the mechanisms of protective immunity to malaria. If parasite density was >2500 rings/\mu L of blood for patients in Pikine and Ndiop and >15,000 rings/\mu L for patients in Dielmo, then 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 within 84 h. Thin blood smears were stained using an RAL kit (Réactifs RAL, Paris, France) and examined to determine parasite density and confirm monoinfection by \emph{P. falciparum}. Samples with parasitaemia ranging from 0.01% to 9.4% were used to test drug sensitivity. Parasitized erythrocytes were washed three times in RPMI 1640 medium (Gibco BRL, Paisley, UK) and supplemented with 10% human serum (pooled from different A ~ or AB, non-immune donors outside of the area of malaria endemicity) and buffered with 25 mM HEPES and 25 mM NaHCO\_3.

\textbf{Drugs}

Chloroquine diphosphate, quinine hydrochloride and amodiaquine dihydrochloride were obtained from Sigma Chemical Co. (St Louis, MO, USA) and pyronaridine phosphate from the World Health Organization (WHO; batch no. 210642). Stock solutions were prepared in sterile distilled water for chloroquine diphosphate, pyronaridine phosphate and amodiaquine dihydrochloride, and in methanol for quinine (methanol had no cytotoxic effect on parasite growth at the dilution used and there was no evidence of precipitation of antimalarials when dilutions were made in water). Two-fold serial dilutions were prepared in sterile distilled water. Final concentrations, ranging from 25 to 3200 nM for chloroquine, 50 to 3200 nM for quinine, 3.1 to 400 nM for amodiaquine and 0.8 to 100 nM for pyronaridine, were distributed in triplicate into Falcon 96-well flat-bottomed plates.

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

\textbf{In-vitro assay}

For in-vitro isotopic microtests, the suspension of parasitized erythrocytes was distributed at 200 \mu L/well in 96-well plates containing antimalarial agents. Parasite growth was assessed by adding 1 \mu Ci of \[^{3}H\]\hypoxanthine with a specific activity of 14.1 Ci/mmol (NEN Products, Dreieich, Germany) to each well. Plates were incubated for 42 h at 37°C in an atmosphere of 10% O\(_2\), 6% CO\(_2\), 84% N\(_2\) and a humidity of 95% (optimum conditions in our laboratory). Immediately after incubation, the plates were frozen and then thawed to lyse erythrocytes. The contents of each well were collected on standard filter microplates (U nifilter GF/B, Packard Instrument Company, Meriden, CT, USA) and washed using a cell harvester (FilterMate Cell Harvester, Packard). Filter microplates were dried and 25 \mu L of scintillation cocktail (Microscint O, Packard) was placed in each well. Radioactivity incorporated by the parasites was measured using a scintillation counter (Top Count, Packard).

The 50\% inhibitory concentration (IC\(_{50}\)), i.e. the drug concentration corresponding to uptake of 50\% of the \[^{3}H\]\hypoxanthine by the parasites in drug-free control wells, was determined by non-linear regression analysis of log-dose/response curves. Data were expressed as the geometric mean IC\(_{50}\) and 95\% confidence intervals (95\% CI) were calculated. The unpaired \(t\)-test was used to compare IC\(_{50}\) values from chloroquine-susceptible and chloroquine-resistant isolates. The degree of cross-resistance of standard antimalarials with Mannich bases was estimated by calculating the Pearson correlation coefficient (\(r\)) and coefficient of determination (\(r^2\)). Isolates were considered as chloroquine-resistant if the IC\(_{50}\) was >100 nM. Cut-off values for resistance to amodiaquine and quinine were 80 nM and 500 nM, respectively. The in-vitro threshold value of antimalarials has been defined statistically (>2 s.d. above the mean). Only in-vitro resistance to chloroquine has been confirmed to correlate with therapeutic effectiveness in vivo. The cut-off for pyronaridine-resistance has not been determined.
Results

Numbers of isolates of *P. falciparum* successfully cultured in the tests were 158/161 for chloroquine and 161/161 for the other antimalarial drugs.

The IC\textsubscript{50} values for pyronaridine were in the range 0.8–34.6 nM (mean IC\textsubscript{50}, 3.8 nM; 95% CI, 3.1–4.4 nM). The IC\textsubscript{50} values for amodiaquine were in the range 6.2–42.1 nM (mean IC\textsubscript{50}, 12.0 nM; 95% CI, 10.0–14.0 nM). Based on our criterion (IC\textsubscript{50} > 100 nM), 78/158 fresh isolates of *P. falciparum* studied were considered to be chloroquine-resistant (mean IC\textsubscript{50}, 245 nM, 95% CI, 221–270 nM) (Table I). As shown in Table I, pyronaridine and amodiaquine were less active against chloroquine-resistant isolates (P < 0.002 and P < 0.025, respectively).

Based on statistical calculation using the present data (mean IC\textsubscript{50} + 2 s.d.), the cut-off value for in-vitro susceptibility to pyronaridine is IC\textsubscript{50} < 15 nM; eight isolates (5%) showed an IC\textsubscript{50} of >15 nM. Pyronaridine and amodiaquine were both highly effective against all isolates tested (Figure), while 51% and 10% of the isolates showed either resistance or reduced susceptibility to chloroquine and quinine, respectively.

Significant positive correlations, suggesting in vitro cross-resistance among these drugs, are shown in Table II.

Discussion

This area of Senegal is known for *P. falciparum* with in-vitro chloroquine-resistance and reduced susceptibility to cycloguanil, mefloquine and halofantrine. Pyronaridine was highly effective against all isolates tested, though many isolates showed resistance to chloroquine (51%) or reduced susceptibility to quinine (10%). The present study confirmed the high level of in-vitro antimalarial activity of pyronaridine reported by other workers using field isolates or culture-adapted strains of *P. falciparum* from Asia or Africa using the WHO microtest technique (values between 10 and 20 nM) or using isotopic semimicrotest (values between 6 and 10 nM). Nevertheless, our mean IC\textsubscript{50} value of 3.8 nM was lower.

There are conflicting reports of pyronaridine activity against chloroquine-resistant and chloroquine-susceptible strains of *P. falciparum*. Childs et al. showed that pyronaridine appeared to be equally effective in vitro against 37 isolates from areas of Thailand with different chloroquine-resistance levels and against two reference clones, chloroquine-susceptible D6 (Sierra Leone) and chloroquine-resistant W2 (Indochina). Basco & Le Bras showed no correlation between resistance to pyronaridine and chloroquine in 31 isolates from Central and West Africa. In the present study, we found that pyronaridine was significantly less active against chloroquine-resistant isolates (P < 0.002). The positive correlation found between the chloroquine and pyronaridine responses (r = 0.44, P < 0.001, Table I), confirmed previous findings.

Amodiaquine was highly effective against all isolates tested (mean IC\textsubscript{50}, 12.0 nM; 95% CI, 10.0–14.0 nM). No in-vitro resistance to amodiaquine was observed among these isolates from Senegal. This result confirmed previous studies which showed low levels of resistance to amodiaquine in Central Africa. In the present study, we found that pyronaridine was significantly less active against chloroquine-resistant parasites in vitro even if, as also observed in this study, amodiaquine frequently shows cross-resistance with chloroquine in vitro.

The mechanism of action of chloroquine and the mechanism of resistance to it are not well understood. It

<table>
<thead>
<tr>
<th>Drug pair</th>
<th>r</th>
<th>r²</th>
<th>P</th>
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<tbody>
<tr>
<td>Pyronaridine/quinine</td>
<td>+0.66</td>
<td>0.44</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pyronaridine/amodiaquine</td>
<td>+0.58</td>
<td>0.34</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pyronaridine/chloroquine*</td>
<td>+0.44</td>
<td>0.19</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Amodiaquine/quinine</td>
<td>+0.45</td>
<td>0.21</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Amodiaquine/chloroquine*</td>
<td>+0.38</td>
<td>0.14</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Pearson correlation coefficient; n = 161 except for the asterisked combinations, for which it was 158.

Table I. In-vitro susceptibility of 158 *African* isolates (Senegal) of *Plasmodium falciparum* to pyronaridine, amodiaquine, chloroquine and quinine

<table>
<thead>
<tr>
<th>Drug</th>
<th>Chloroquine-susceptible isolates (n = 80)</th>
<th>Chloroquine-resistant isolates (n = 78)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC\textsubscript{50} (nM) means 95% confidence limits</td>
<td>IC\textsubscript{50} (nM) means 95% confidence limits</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>35.7 30.2–40.9</td>
<td>245.1 220.7–269.5</td>
</tr>
<tr>
<td>Pyronaridine</td>
<td>2.9 2.3–3.5</td>
<td>4.9 3.8–6.0</td>
</tr>
<tr>
<td>Amodiaquine</td>
<td>9.6 6.1–13.1</td>
<td>14.3 11.9–16.7</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.
has been hypothesized that, as a weak base, chloroquine and its close analogues, follows the pH gradient and accumulates in the food vacuole of susceptible parasites. Chloroquine may interfere with haemoglobin degradation in food vacuoles by raising the vacuolar pH and/or inhibiting haem polymerase. Resistant parasites actively expel chloroquine, probably by means of a transporter encoded by a multidrug-resistance (mdr) gene. It seems likely, therefore, that chloroquine-resistance is caused by mutations in a number of genes. Wellems et al. have produced evidence from linkage analysis of a P. falciparum cross that a gene located on chromosome 7 determines the resistance. Ultrastructural studies suggest that pyronaridine also accumulates in the food vacuole and impairs haemoglobin degeneration. The high activity of pyronaridine against chloroquine-resistant parasites could be the result of a higher affinity of pyronaridine for the enzyme haem polymerase or a lower affinity for the putative chloroquine efflux pump.

Accumulation of amodiaquine was correlated with accumulation of chloroquine and accumulation of both drugs was significantly reduced in chloroquine-resistant isolates. 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. The contribution of this polymorphism to amodiaquine resistance has been also assessed, and may explain cross-resistance between these drugs. There are conflicting reports on cross-resistance between pyronaridine, quinine and amodiaquine. In a study of 37 Asian isolates, pyronaridine-resistant isolates were not cross-resistant to quinine or amodiaquine. We noted positive correlations between the parasite responses to pyronaridine and quinine (44% of isolates), to pyronaridine and amodiaquine (34%), and to amodiaquine and quinine (21%), confirming a previous study.

Despite the positive correlations between responses of 161 P. falciparum isolates to pyronaridine, amodiaquine,
chloroquine and quinine, the high activity of pyronaridine and amodiaquine is evident. Pyronaridine and amodiaquine may be important alternative drugs for treating chloroquine-resistant malaria. However, in-vitro cross-resistance reinforces the idea that novel antimalarials should not be used as monotherapy. There is an urgent need to find a rational partner compound with which pyronaridine can be administered in order to prolong its potentially useful life; this should ideally be done before and not after resistance has begun to emerge. One possible candidate for this partner is arteether. Synergy between pyronaridine and arteether was observed against rodent parasites. A arteether showed high in-vitro activity against isolates from this area of Senegal. In addition, pyronaridine is well-tolerated, and its side effects are mild. However, if pyronaridine is to be developed further, costs will need very careful attention. Further pharmacokinetic and clinical studies in different geographical regions are needed to define the therapeutic role of pyronaridine.

Amodiaquine seems to be no more toxic than chloroquine or sulphadoxine/pyrimethamine when administered at doses up to 35 mg/kg total dose over 3 days for treating adults and children with uncomplicated malaria. Idiosyncratic agranulocytosis, which is observed from time to time with amodiaquine, may be associated with chemical modifications of Mannich antimalarial agents. If Mannich bases are used for treating malaria, it will be important to monitor continuously the response of parasite populations.

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References

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