IN VITRO ACTIVITY OF ARTEMETHER AGAINST AFRICAN ISOLATES (SENEGAL) OF Plasmodium falciparum IN COMPARISON WITH STANDARD ANTIMALARIAL DRUGS

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Abstract. The in vitro activity of artemether against 56 African isolates of Plasmodium falciparum from Senegal was evaluated using an isotope-based drug susceptibility semi-microtest. The 50% inhibitory concentration (IC50) values for artemether were in a narrow range from 0.8 to 15.2 nM (mean IC50 = 3.43 nM) and the 95% confidence interval (CI) was 2.50–4.36 nM. Artemether was equally effective on chloroquine-sensitive and chloroquine-resistant isolates (mean IC50 = 346 nM, 95% CI = 2.08–4.84 nM versus mean IC50 = 2.80 nM, 95% CI = 2.00–3.60 nM). There was a significant positive correlation between responses to artemether and mefloquine (r² = 0.36, P < 0.001), artemether and quinine (r² = 0.085, P < 0.05), artemether and halofantrine (r² = 0.075, P < 0.05), quinine and mefloquine (r² = 0.205, P < 0.01), quinine and halofantrine (r² = 0.124, P < 0.05), and mefloquine and halofantrine (r² = 0.801, P < 0.001). A positive correlation between these drugs suggests in vitro cross-resistance or at least in vitro cross-susceptibility.

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. For this reason, the increasing prevalence of strains of Plasmodium falciparum resistant to chloroquine and other antimalarial drugs poses a serious problem for control of malaria. There is an urgent need to find and develop alternative drugs against chloroquine-resistant Plasmodium falciparum.

One group of alternative antimalarial drugs comprises artesinin (qinghaosu) and its derivatives. Artesinin is a traditional Chinese medicinal herb derived from Artemisia annua. It has a sesquiterpene lactone with a peroxide bridge and antimalarial activity has been attributed to the peroxy moiety by a mechanism involving production of free radicals that damage the parasite membrane. Artemether (an oil-soluble artesinin derivative) has shown effectiveness against chloroquine-resistant strains of P. falciparum in China and Thailand. However, little information is available concerning the in vitro activity of artesinin and its derivatives against isolates of P. falciparum from Africa other than reference strains.

The two-fold aim of this in vitro study was to evaluate the activity of artemether against fresh isolates of P. falciparum from Senegal, and to analyze cross-susceptibility between artemether, chloroquine, quinine, mefloquine, and halofantrine.

MATERIALS AND METHODS

Isolates of P. falciparum. All programs were reviewed and approved by the Conseil de Perfectionnement of the Institut de Dakar, which is presided over by the Senegalese Health Secretary. Informed oral consent was obtained from the patients and/or their parents before collection of blood samples. Only oral consent was obtained because only 5% of the people in Dielmo, Ndiop, and Toubakouta can read and write. Between October 1995 and January 1996, 56 fresh P. falciparum isolates were prepared from samples obtained after oral consent in Dielmo, Ndiop, and Toubakouta (280 km southeast of Dakar) in the Fatick region of Senegal. Patients from Toubakouta were recruited at the public center 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 the parasite density was greater than 2,500 rings/μl of blood for patients in Toubakouta and Ndiop (seasonal transmission) and greater than 15,000 rings/μl for patients in Dielmo (continuous transmission), then venous blood was collected before treatment in Vacutainer® ACD tubes (Becton Dickinson, Rutherford, NJ) and transported at 4°C to our laboratory in Marseille within 8 hr. Thin blood smears were stained using an RAL® kit (Reactifs RAL, Paris, France) and examined to determine parasite density and confirm monoinfection by P. falciparum. Samples with parasitemias ranging from 0.1% to 8.0% 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%. Parasite growth was assessed after 42 hr of incubation before reinvasion. Susceptibility to chloroquine, quinine, mefloquine, halofantrine, and artemether was determined after suspension of the erythrocytes in RPMI 1640 medium supplemented with 10% human serum (pooled from different A or AB nonimmune donors who were from outside the area of malaria endemicity) and buffered with 25 mM HEPES and 25 mM NaHCO3.

Plasma measurements of the relevant antimalarials were evaluated by high-performance liquid chromatography. Traces of chloroquine were found in the plasma of 70% of the patients, but there was no evidence that traces of antimalarials in infected erythrocytes after three washings of the blood in RPMI 1640 medium affected the in vitro assays.

Drugs. Chloroquine diphosphate and quinine hydrochloride were obtained from Sigma Chemicals (St. Louis, MO), mefloquine hydrochloride from Roche (Paris, France), halofantrine hydrochloride from Smith-Kline Beecham (Hertfordshire, United Kingdom), and artemether from Rhone Poulenc Rorer (Antony, France). Stock solutions were pre-
pared in sterile distilled water for chloroquine diphosphate and in methanol for the other antimalarial agents. Previous studies in our laboratory indicated that methanol had no cytotoxicity on parasite growth at the lowest dilution (methanol/water) used with the highest final concentration of antimalarial. There was no evidence of precipitation of antimalariais when dilutions were made in water. Two-fold serial dilutions were prepared in sterile distilled water and distributed in triplicate into 24-well flat-bottomed plates (no. 13124; Nunc, Roskilde, Denmark). Final concentrations, which ranged from 25 to 3,200 nM for chloroquine, 50 to 3,200 nM for quinine, 2.5 to 400 nM for mefloquine, 0.25 to 32 nM for halofantrine, and 0.8 to 100 nM for artemether, were prepared in triplicate in 24-well plates.

The chloroquine-susceptible *P. falciparum* D6 clone (Sierra Leone) and the chloroquine-resistant W2 clone (Indonesia) were used as references to test each batch of plates. Plate batches with an in vitro response 15–20% below or above the mean of the relevant antimalarias were eliminated. Reference clones were maintained in continuous culture and were synchronized twice with sorbitol.

**In vitro assay.** The in vitro semi-microtest used in the present study has been previously described by Le Bras and Deloron. The suspension of parasitized erythrocytes (750 µl/well) was distributed in 24-well plates predosed with antimalarial agents. Plates were incubated for 42 hr at 37°C in an atmosphere of 10% O₂, 6% CO₂, 84% N₂, and a humidity of 95%. These had been previously established as optimum conditions in our laboratory. Parasite growth was assessed after 18 hr by adding 1 mCi of ³H-hypoxanthine with a specific activity 14.1 Ci/mmol (New England Nuclear Products, Dreieich, Germany) to each well. Immediately after incubation, the plates were frozen and thawed to lyse the erythrocytes. The contents of each well were collected on standard filter microplates (UniFilter™ CF/B; Packard Instrument Co., Meriden, CT) and washed using a cell harvester (FilterMate® Cell Harvester, Packard Instrument Co.). Filter microplates were dried and 100 µl of scintillation cocktail (Microscint® O; Packard Instrument Co.) was placed in each well. Radioactivity incorporated by the parasites was measured using a scintillation counter (Top Count®, Packard Instrument Co.).

The 50% inhibitory concentration (IC₅₀), i.e., the drug concentration corresponding to 50% of the uptake of ³H-hypoxanthine by the parasites in drug-free control wells, was determined by nonlinear regression analysis of log-dose/response curves. Isolates were considered chloroquine-resistant if the IC₅₀ was greater than 100 nM. Cut-off values for resistance to quinine, mefloquine, and halofantrine were 500 nM, 20 nM, and 5 nM, respectively. The in vitro threshold values for antimalariais have been defined statistically (>2 SD above the mean). In vitro resistance to chloroquine was evaluated statistically and by correlation between in vitro response and therapeutic effectiveness. The cut-off value for artemether resistance was not determined because until now only in vitro susceptibility of reference clones or of a restricted number of isolates had been determined. These data were not enough to evaluate statistically the in vitro resistance cut-off value. Data were expressed as the geometric mean IC₅₀ and 95% confidence intervals (95 CIs) were calculated. The unpaired t-test was used to compare IC₅₀ values from chloroquine-susceptible and chloroquine-resistant isolates. Assessment of standard cross-resistance of other antimalariais with artemether was estimated by the Pearson correlation coefficient (r) and the coefficient of determination (r²).

**RESULTS**

The IC₅₀ values for artemether were in a narrow range from 0.8 to 15.2 nM (mean IC₅₀ = 3.43 nM) and the 95% CI was 2.50–4.36 nM (Figure 1). Based on our criterion (IC₅₀ > 100 nM), 18 of the 56 fresh isolates of *P. falciparum* studied were considered as chloroquine-resistant (mean IC₅₀ = 179 nM, 95% CI = 132–226 nM) (Table 1). As shown in Table 1, the IC₅₀ values for artemether obtained with chloroquine-sensitive isolates and chloroquine-resistant isolates were similar (mean IC₅₀ = 3.46 nM, 95% CI = 2.08–4.84 nM versus mean IC₅₀ = 2.80 nM, 95% CI = 2.00–3.60 nM).

A significant positive correlation was observed between the responses to artemether and mefloquine (r² = 0.360, P < 0.001), artemether and quinine (r² = 0.085, P < 0.05), artemether and halofantrine (r² = 0.075, P < 0.05), quinine and mefloquine (r² = 0.205, P < 0.01), quinine and halofantrine (r² = 0.124, P < 0.05), and mefloquine and halofantrine (r² = 0.801, P < 0.001) (Table 2). A significant negative correlation was observed between the responses to chloroquine and mefloquine (r² = 0.137, P < 0.05).

| **Table 1** In vitro response of 50 African isolates of *Plasmodium falciparum* from Senegal to chloroquine and artemether* |
| **Drug** | **IC₅₀ (nM)** | **95% confidence intervals** | **IC₅₀ (nM)** | **95% confidence intervals** |
| Chloroquine-sensitive isolates (n = 32) | Chloroquine-resistant isolates (n = 18) |
| Chloroquine | 41.0 | 32.4–49.6 | 179.3 | 132.4–226.2 |
| Artemether | 3.46 | 2.08–4.84 | 2.8 | 2.01–3.59 |

* Values are geometric mean 50% inhibitory concentrations (IC₅₀) in nanomoles/liter.
TABLE 2
Correlation in vitro responses of African isolates of *Plasmodium falciparum* from Senegal to artesether and standard antimalarial drugs

<table>
<thead>
<tr>
<th>Drug pair</th>
<th>Number of isolates</th>
<th>Correlation coefficient $r$</th>
<th>Coefficient of determination $r^2$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artemether</td>
<td>50</td>
<td>-0.172</td>
<td>0.030</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>Artemether</td>
<td>52</td>
<td>0.291</td>
<td>0.085</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Artemether</td>
<td>31</td>
<td>0.600</td>
<td>0.360</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Artemether</td>
<td>50</td>
<td>0.273</td>
<td>0.075</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>49</td>
<td>0.008</td>
<td>&lt;0.001</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>31</td>
<td>-0.370</td>
<td>0.137</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>48</td>
<td>-0.143</td>
<td>0.020</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>Quinine</td>
<td>31</td>
<td>0.453</td>
<td>0.205</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Quinine</td>
<td>49</td>
<td>0.352</td>
<td>0.124</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>30</td>
<td>0.895</td>
<td>0.801</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The cut-off value for resistance to artesether remains unclear. Based on statistical calculations using the present data (mean IC$_{50}$ + 2 SD), the cut-off value for resistance to artesether is an IC$_{50}$ value greater than 10.5 nM. This is consistent with a value of 10.0 nM previously estimated for resistance of African isolates to artesether (Le Bras J, unpublished data). However, artesether has been shown to be effective in vivo and no therapeutic failure of artesether has been reported. The six isolates in this study with IC$_{50}$ values greater than 10.5 nM were not considered resistant. Accurate determination of the IC$_{50}$ cut-off value for resistance will require correlation between responses observed in vitro and results obtained after chemotherapy in vivo.

In the present study, the in vitro response of the chloroquine-susceptible and chloroquine-resistant isolates to artesether did not differ significantly. This finding is in contrast with previous studies in Central and West Africa and in Cameroon, which showed that susceptibility to artesether was inversely related to susceptibility to chloroquine.

Also in contrast with previous findings indicating negative or weakly positive correlations between responses to artemisinin and chloroquine, the present study showed no significant correlation. On the other hand, our data did suggest a positive correlation between in vitro responses to artesether, mefloquine, quinine, and halofantrine. Nevertheless, the coefficient of determination ($r^2$) values for the correlations between artesether and the aminoalcohols are minimal to medium: 0.075 for artesether/halofantrine, 0.085 for artesether/quinine, and 0.36 for artesether/mefloquine. Although these positive correlations are in agreement with previous reports in Africa, it should be emphasized that a positive correlation in vitro is not necessarily predictive of cross-resistance in vivo. In clinical practice, halofantrine seems to be effective against mefloquine-resistant isolates despite a positive correlation between the IC$_{50}$ values of halofantrine and mefloquine in 80 of 100 isolates. However, reduced in vitro susceptibility to mefloquine and halofantrine and treatment failures with halofantrine and mefloquine, despite therapeutically effective plasma concentrations, have been reported in west African patients living in areas where halofantrine and mefloquine have not been used extensively (in comparison with quinine, which has been widely used in these areas). Cross-resistance may explain failures with mefloquine and halofantrine. A study prior to the use of mefloquine in Cameroon showed a correlation between mefloquine and quinine resistance. This finding could indicate that some *P. falciparum* isolates are innate less susceptible to mefloquine and halofantrine or that there is cross-resistance between these aminoalcohols.

Since the mechanisms underlying the antimalarial action of artesether, mefloquine, quinine, and halofantrine are still unclear, one can only speculate about the positive, even minor, correlation between responses to artesether and aminoalcohols (especially mefloquine). Several findings suggest that heme may be the common link between these antimalarials. Indeed, heme or heme polymerase in food vacuole has been reported to be the specific target of aminoalcohols. Based on their data, Meshnick and others attributed antimalarial action to a free-radical-generating adduct formed between artemisinin and heme produced by the parasite-induced breakdown of hemoglobin in the erythrocyte. Artemisinin enhances heme-mediated inhibition of membrane-bound enzymes via oxidation of thiol groups on the enzymes. Another possible target for artemisinin and mefloquine is membrane phospholipids. The interaction between the erythrocyte membrane and artemisinin may involve the formation of a covalent linkage with the peroxide bridge, leading to disruption of membrane structure.

The correlation between quinine, mefloquine, and halofantrine may be explained by molecular biology. Although the genetic basis of resistance to various antimalarial drugs has not yet been established, it has been reported that the *P. falciparum* multidrug resistance (*Pfmdr1*) gene and its protein product were amplified and overexpressed in chloroquine-resistant cloned isolates subjected to mefloquine selection pressure. The same investigators also demonstrated decreased resistance to chloroquine, as well as increased resistance to quinine and halofantrine.

In Thailand, where *P. falciparum* is highly resistant to chloroquine and increasingly resistant to quinine and even mefloquine, Luxemburger and others showed that possible oral artesunate at standard doses was superior to intravenous quinine for treatment of uncomplicated multidrug-resistant malaria in patients at high risk of developing life-threatening complications. Several recent studies have confirmed that the combination of artemisinin-related compounds with mefloquine is highly effective even against mefloquine-resistant *P. falciparum*.

Because of its effectiveness against chloroquine-resistant *P. falciparum* malaria, artesether can be considered as a treatment of choice for multidrug-resistant malaria. In addition to effectiveness, two other advantages of artesether treatment are simplicity to administer and good tolerance with no evidence of local toxicity and detectable adverse systemic effects. However, if confirmed in vivo, the correlation, even if minor, observed in the present in vitro study between responses of *P. falciparum* isolates to artesether, quinine, mefloquine, and halofantrine needs investigation. Nevertheless, for the time being, this may not limit the usefulness of artesether as a replacement drug for quinine in Africa.
Acknowledgments: We thank F. Niaye, L. V. Faye, Dr. F. Dienne-Sarr, Dr. A. B. Ly, A. Badiane, C. Bouganali, J. Faye, M. Gning, E. H. Mbengue, O. Sarr, B. Thiam, and Y. Sonko for technical assistance and availability in the field work, D. Fatome for help in transporting isolates, and the staff of the Institut de Medecine Tropicale du Service de Sante des Armees (R. Ges, D. Ramarlah, Y. Troulemans, and D. Ragot) for technical support.

Financial support: This work was supported by the French Ministere de la Cooperation et du Developpement and the Direction Centrale du Service de Sante des Armees.


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