IN VITRO ACTIVITY OF DIHYDROARTESINISIN AGAINST CLINICAL ISOLATES OF PLASMODIUM FALCIPARUM IN YAOUNDE, CAMEROON

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Abstract. The in vitro activities of dihydroartesinin (the biologically active metabolite of artemisinin derivatives), chloroquine, monodesethylamodiaquine (the biologically active metabolite of amodiaquine), quinine, mefloquine, halofantrine, and pyrimethamine were assessed in 65 African isolates of Plasmodium falciparum from Yaoundé, Cameroon, using an isotopic microtest. The 50% inhibitory concentration (IC₅₀) values for dihydroartesinin were within a narrow range from 0.25 to 4.56 nM, with a geometric mean of 1.11 nM (95% confidence interval = 0.96–1.28 nM). Dihydroartesinin was equally active (P > 0.05) against the chloroquine-sensitive isolates (geometric mean IC₅₀ = 1.25 nM, 95% confidence interval = 0.99–1.57 nM) and the chloroquine-resistant isolates (geometric mean IC₅₀ = 0.979 nM, 95% confidence interval = 0.816–1.18 nM). A significant positive correlation was observed between the responses to dihydroartesinin and mefloquine (r = 0.662) or halofantrine (r = 0.284), suggesting in vitro cross-resistance. There was no correlation between the responses to dihydroartesinin and other antimalarial drugs.

Artemisinin (qinghaosu) has been used for centuries in traditional Chinese medicine for antimalarial treatment. In 1972, Chinese physicians isolated artemisinin from the plant Artemisia annua. Artemisinin is a sesquiterpene lactone characterized by the presence of an endoperoxide that is associated with a potent antimalarial activity. Because of its chemical instability and poor solubility in water, chemical derivatives have been developed by adding an ether, ester, or other substituents to the hydroxyl group of dihydroartemisinin. Several derivatives, sodium artesunate and artelinic acid, and the oil-water-soluble derivatives, artemether and arteether, have been developed for clinical use. Dihydroartemisinin is more active than the other artemisinin derivatives and that it is well absorbed in the gut. Initial clinical studies have shown that dihydroartemisinin is a highly active antimalarial with rapid and sustained efficacy of oral administration. However, because of the high recrudescence rate when used as monotherapy, many current therapeutic regimens used in Southeast Asia include a combination of chloroquine or pyrimethamine, desferrioxamine, and lumefantrine. In the emergency treatment of severe and complicated falciparum malaria, parenteral administration of dihydroartemisinin has been recommended. Our study revealed no correlation between the responses to dihydroartemisinin and mefloquine or halofantrine, indicating that dihydroartemisinin is a potential antimalarial drug.
...pleas with a monoinfection due to *P. falciparum* and a parasite density > 0.2% from patients whose urine test result was negative were used in this study. *In vitro* assays were performed within 4 hr after blood extraction. The patients were treated with amodiaquine, sulfadoxine-pyrimethamine, or quinine. This study was approved by the Cameroonian National Ethics Committee.

**Drugs.** Antimalarial drugs used in this study were obtained from the following sources: chloroquine diphosphate, quinine hydrochloride, and pyrimethamine base (Sigma Chemical Company, St. Louis, MO); monodesethylamodiaquine hydrochloride (the biologically active human metabolite of amodiaquine) and dihydroartemisinin (SAce Fine Chemicals, Lugano, Switzerland) through the courtesy of Dr. Piero Olliaro (Tropical Diseases Research, World Health Organization, Geneva, Switzerland); mefloquine hydrochloride (Hoffmann-La Roche, Basel, Switzerland); and halofantrine hydrochloride, SmithKline Beecham (Hertfordshire, United Kingdom). Stock solutions of chloroquine and monodesethylamodiaquine were prepared in sterile distilled water. Stock solutions of quinine, mefloquine, halofantrine, and dihydroartemisinin were prepared in methanol. The stock solution of pyrimethamine was prepared in ethanol. Two-fold (4-fold for pyrimethamine) serial dilutions of the stock solutions were made in distilled water. The final concentrations ranged from 25 to 1,600 nM for chloroquine and quinine, 1.25-160 nM for monodesethylamodiaquine, 2.5-400 nM for mefloquine, 0.25-32 nM for halofantrine, 0.5-64 nM for dihydroartemisinin, and 0.39-51,200 nM for pyrimethamine. Each concentration was distributed in triplicate in 96-well tissue culture plates.

**In vitro assay.** The venous blood samples were washed three times in RPMI 1640 medium. The erythrocytes were resuspended in the complete RPMI 1640 medium consisting of 10% human serum (from non-immune European donors without previous history of malaria), 25 mM HEPES buffer, and 25 mM sodium bicarbonate at a hematocrit of 1.5% and an initial parasitemia of 0.2-1.0%. The p-aminobenzoic acid and folic acid-free RPMI 1640 medium was used to assess the *in vitro* sensitivity to pyrimethamine. If the blood sample had a parasitemia > 1.0%, fresh uninfected, type A+ erythrocytes were added to adjust the parasitemia to 0.2% from patients whose urine test result was negative. The parasites were incubated at 37°C in 5% CO₂ for 18 hr. To assess parasite growth, 3H-hypoxanthine (specific activity = 16.3 Ci/mmol; Amersham, Buckinghamshire, United Kingdom) was added. After an additional 24 hr of incubation (48 hr for pyrimethamine), the plates were frozen to terminate the *in vitro* assay. The plates were thawed, and the contents of each well were collected on glass-fiber filter papers, washed, and dried using a cell harvester. The filter disks were transferred into scintillation tubes, and 2 ml of scintillation cocktail (Organic Counting Scintillator®; Amersham) were added. The incorporation of 3H-hypoxanthine was quantitated using a liquid scintillation counter (Wallac 1410; Pharmacia, Uppsala, Sweden).

**Data analysis.** The 50% inhibitory concentration (IC₅₀) values, defined as the drug concentration corresponding to

50% of the uptake of ³H-hypoxanthine measured in the drug-free control wells, were determined by nonlinear regression analysis of logarithm of concentrations plotted against growth inhibition. Prism® software (GraphPad Software, Inc., San Diego, CA) was used to obtain the best-fitting sigmoid curve, and the IC₅₀ values were calculated using the logistic equation. The threshold IC₅₀ values for *in vitro* resistance to chloroquine, monodesethylamodiaquine, quinine, mefloquine, and halofantrine were estimated to be > 100 nM, > 60 nM, > 800 nM, > 30 nM, and > 6 nM, respectively. The resistance levels of pyrimethamine (for a 72-hr incubation) and dihydroartemisinin are still undefined.

Data were expressed as geometric mean IC₅₀ values and the 95% confidence intervals. The mean logarithmic IC₅₀ values of various drugs were compared between the chloroquine-sensitive and the chloroquine-resistant parasites using the Student’s unpaired t-test. Correlation of the IC₅₀ values for different drugs was calculated by Spearman’s rank-order correlation test. The significance level was fixed at 0.05. Data were analyzed with the Statview software (Abacus Concepts, Inc., Calabasas, CA).

**RESULTS**

The *in vitro* activities of chloroquine, monodesethylamodiaquine, quinine, mefloquine, halofantrine, pyrimethamine, and dihydroartemisinin were assessed in 65, 30, 60, 25, 58, 34, and 65 isolates, respectively. Dihydroartemisinin was the most potent drug (geometric mean IC₅₀ = 1.11 nM, 95% confidence interval = 0.96-1.28 nM, range = 0.25-4.56 nM, n = 65) among the compounds tested in this study (Figure 1). Dihydroartemisinin was almost twice as active as halofantrine (geometric mean IC₅₀ = 1.99 nM, 95% confidence interval = 1.70-2.34 nM) and 10 times more potent than mefloquine (geometric mean IC₅₀ = 10.8 nM, 95% confidence interval = 8.12-14.5 nM). The *in vitro* activities of the antimalarial drugs and the number of chloroquine-sensitive and chloroquine-resistant isolates tested for each drug are summarized in Table 1. Monodesethylamodiaquine and quinine were more active (*P* < 0.05) against the chloro-

![FIGURE 1. Distribution of the 50% inhibitory concentrations (IC₅₀) in nanomoles of *Plasmodium falciparum* isolates from Cameroon for chloroquine (CQ), monodesethylamodiaquine (mAQ), quinine (QU), halofantrine (HF), mefloquine (MQ), pyrimethamine (PYR), and dihydroartemisinin (DHA).](image-url)
**IN VITRO ACTIVITY OF DIPHARARTEMISININ**

**Table 1**

*In vitro* response of chloroquine-sensitive and chloroquine-resistant Cameroonian isolates of *Plasmodium falciparum*.  

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<thead>
<tr>
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<th>Chloroquine-sensitive isolates</th>
<th>Chloroquine-resistant isolates</th>
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<td>Dihydroartemisinin</td>
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<td>Chloroquine</td>
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Values are geometric mean inhibition concentrations (IC₅₀) in nanomoles per liter. 

*DISCUSSION*

In *in vitro* and *in vivo* studies, the antimalarial activity of several drugs has been evaluated against Cameroonian isolates of *Plasmodium falciparum*. The data presented in Table 1 show the geometric mean IC₅₀ values for a selection of drugs against chloroquine-sensitive and chloroquine-resistant isolates.

The geometric mean IC₅₀ values for dihydroartemisinin were significantly lower against chloroquine-resistant parasites compared to chloroquine-sensitive isolates (unpaired *t*-test: *P* < 0.05). This indicates that dihydroartemisinin was more active against chloroquine-resistant parasites than against chloroquine-sensitive isolates.

In contrast, chloroquine was more active against chloroquine-sensitive isolates. Pyrimethamine showed activity similar to chloroquine against both chloroquine-sensitive and chloroquine-resistant isolates.

Monodesethylamodiaquine and quinine were active against chloroquine-resistant parasites, with monodesethylamodiaquine showing a widespread range of IC₅₀ values (0.24-8,940 nM). Quinine had IC₅₀ values (623 nM) that were not significantly different from those of chloroquine and monodesethylamodiaquine (*r* = 0.423).

The responses of Cameroonian isolates to dihydroartemisinin and chloroquine, monodesethylamodiaquine, pyrimethamine, and quinine were correlated, with significant correlation (*r* = 0.405) between the responses to dihydroartemisinin and chloroquine, monodesethylamodiaquine, pyrimethamine, and quinine.

In the case of chloroquine-resistant parasites, none of the isolates showed a complete response to quinine, with all isolates possessing mutant alleles associated with sulfadoxine resistance. Pyrimethamine was also shown to be inactive against chloroquine-resistant parasites.

**REFERENCES**

1. Ringwald M, unpublished data. There is no documented case of quinine resistance in the studied population

2. A significant negative correlation was observed between the responses of Cameroonian isolates to dihydroartemisinin and chloroquine.

3. Significant correlation (*r* = 0.423) was observed between the responses to dihydroartemisinin and chloroquine-resistant isolates.

4. A significant correlation (*r* = 0.662) was observed between the responses of Cameroonian isolates to dihydroartemisinin and chloroquine-resistant isolates.

5. Significant correlation (*r* = 0.836) was observed between the responses of Cameroonian isolates to dihydroartemisinin and chloroquine-resistant isolates.

6. Significant correlation (*r* = 0.284) was observed between the responses of Cameroonian isolates to dihydroartemisinin and chloroquine-resistant isolates.

7. Significant correlation (*r* = 0.405) was observed between the responses of Cameroonian isolates to dihydroartemisinin and chloroquine-resistant isolates.
failure were ascribed to underdosage. Clinical trials in Yaoundé have also shown that pyronaridine, mefloquine, halofantrine, and arteether are highly effective (Ringwald R unpublished data).

In view of our data on the epidemiology of drug-resistant *P. falciparum*, artemisinin derivatives are currently not essential for malaria control in Yaoundé. We have nevertheless assessed the *in vitro* activity of dihydroartemisinin since arteether and artesunate are commercially available in Cameroon. Compared with other antimalarial drugs, dihydroartemisinin was almost as active as atovaquone against the African isolates (geometric mean IC₅₀ = 0.89 nM [n = 35] and 0.91 nM [n = 26] against the chloroquine-sensitive and the chloroquine-resistant isolates, respectively). These two drugs were the most potent test compounds among the currently available antimalarial drugs. Atovaquone is now being marketed in combination with proguanil with good results of Bustos and others, which showed that of the four artemisinin derivatives (artesunate, artemether, arteether, and dihydroartemisinin) tested against Philippines isolates, artesunate (median IC₅₀ = 0.9 nM) was the most potent in vitro. These results raise the question regarding the choice of artemisinin derivatives for *in vitro* assays. The most reasonable choice seems to be the use of dihydroartemisinin for *in vitro* assays since this derivative is relatively stable and all currently used artemisinin derivatives and those that are under advanced phases of development are rapidly transformed into dihydroartemisinin in humans.

The significant positive correlation between the *in vivo* responses to chloroquine and monodesethylamodiaquine or quinine and between the response to mefloquine and halofantrine is in agreement with *in vivo* resistance to amodiaquine, quinine, and halofantrine observed in Southeast Asia where a high level of resistance to chloroquine and mefloquine has emerged. Cross-resistance between pyrimethamine and cycloguanil has also been observed in *in vitro* and *in vivo*. Cross-resistance patterns between these drugs may be, at least in part, explained on the basis of similar chemical structures. Our study suggests in *vitro* cross-resistance between dihydroartemisinin and aminolevulinic acid (mefloquine and halofantrine), which do not share any similar chemical features. A positive correlation was also obtained with other artemisinin derivatives and aminoalcohols in several *in vitro* studies. The clinical and epidemiologic significance of the *in vitro* cross-resistance between these two classes of drugs is still unknown. The mechanism of action of artemisinin derivatives is thought to involve the interac-
tion between the drugs and hemin within the digestive vac-

erole of the parasite. The iron-dependent decomposition of artesinin generates free oxygen radicals that destroy the parasites. The 4-aminquinolines and aminoalcohols also concentrate within the digestive vacuole but their precise mode of action has not been elucidated. Further studies are needed to understand the in vitro cross-resistance pattern between artesinin and aminoalcohols.


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42. ter Kuile FO, Dolan G, Nosten F, Edstein MD, Luxemburger C, Phaipun L, Chongsuphajaisiddhi T, Webster HK, White NJ,