ANTIBIOTICS FOR PROPHYLAXIS OF PLASMODIUM FALCIPARUM INFECTIONS: IN VITRO ACTIVITY OF DOXYCYCLINE AGAINST SENEGALESE ISOLATES

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Abstract. The in vitro activities of doxycycline, chloroquine, quinine, amodiaquine, artemether, pyrimethamine, and cycloguanil were evaluated against Plasmodium falciparum isolates from Senegal (Dielmo and Ndiop), using an isotopic, micro, drug susceptibility test. The 71 50% inhibitory concentration (IC50) values for doxycycline ranged from 0.7 to 108.0 μM and the geometric mean IC50 for the 71 isolates was 11.3 μM (95% confidence interval = 9.5–13.4 μM). The activity of doxycycline did not differ significantly (P = 0.0858) between the chloroquine-susceptible isolates and the chloroquine-resistant isolates. There was no in vitro correlation between the responses to doxycycline and those to artemether, chloroquine, quinine, amodiaquine, pyrimethamine, and cycloguanil, suggesting no in vitro cross-resistance among these drugs. Potency was increased by prolonged exposure. In 96-hr incubations, the activity of doxycycline was 4–5-fold more increased than in 48-hr incubations. The in vitro activity of doxycycline against intraerythrocytic stages of multidrug-resistant P. falciparum, its action against the preerythrocytic forms, the lack of correlation between the responses in vitro of P. falciparum to doxycycline and the other antimalarial drugs, and its original potential site of action are factors that favor its use as antimarial drug.

The 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 poses a serious problem for control of malaria. Failures of antimalarial prophylaxis with chloroquine, the combination of chloroquine and proguanil, and mefloquine, and clinical failures with halofantrine and quinine showed antimalarial activity of dihydroorotate dehydrogenase of the pyrimidine pathway in P. falciparum, presumably due to inhibition of enzyme protein synthesis. Daily doxycycline has been shown to be an effective causal chemoprophylactic in Thai malaria. The current options for reducing the morbidity and mortality of malaria are chemoprophylaxis and chemotherapy. The aim of the present study was to determine the in vitro activity of doxycycline against P. falciparum isolates and compare this with that of chloroquine, quinine, amodiaquine, artemether, pyrimethamine, and cycloguanil. The activity of doxycycline was assessed for 20 isolates after incubation for 48 hr and 96 hr.

MATERIALS AND METHODS

Isolates of P. falciparum. Between September and November 1998, 104 P. falciparum isolates were prepared from samples obtained in Dielmo and Ndiop (280 km southeast of Dakar) in Fatick region of Senegal. 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. Thrombocytopenic samples were obtained using an RAL kit (Réactifs RAL, Paris, France) and examined to determine parasite density. Samples with parasitemias ranging from 0.01% to 6.0% were used to test drug susceptibility. Parasitized erythrocytes were washed 3 times in RPMI 1640 medium (Life Technologies, 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%. Susceptibilities to doxycycline, amodiaquine, chloroquine, quinine, and artemether were determined after suspension in RPMI 1640 medium and to pyrimethamine and cycloguanil after suspension in RPMI 1640 medium supplemented with reduced p-aminobenzoic acid (0.5 μg/L) and folates (10 μg/L) (Life Technologies). The 2 suspensions were supplemented with 10% human serum (pooled from different A* or AB sera from non-immune donors who did not reside in the area of malaria endemicity) and buffered with 25 mM HEPES and 25 mM NaHCO3. Twenty isolates, collected in the same area from October to December 1997 were used for 48-hr and 96-hr experiments.

Drugs. Doxycycline hydrochloride, chloroquine dihydrochloride, quinine hydrochloride, amodiaquine dihydrochloride, and pyrimethamine were obtained from Sigma (St. Louis, MO), artemether was obtained from Rhône Poulenc Rorer (Antony, France), and cycloguanil was obtained from Zeneca Pharma (Reims, France). Stock solutions were prepared in sterile distilled water for chloroquine dihydrochloride, quinine hydrochloride, amodiaquine dihydrochloride, and pyrimethamine were obtained from Sigma (St. Louis, MO), artemether was obtained from Rhône Poulenc Rorer (Antony, France), and cycloguanil was obtained from Zeneca Pharma (Reims, France). Stock solutions were prepared in sterile distilled water for chloroquine dihydrochloride, quinine hydrochloride, amodiaquine dihydrochloride, and pyrimethamine were obtained from Sigma (St. Louis, MO), artemether was obtained from Rhône Poulenc Rorer (Antony, France), and cycloguanil was obtained from Zeneca Pharma (Reims, France).
amodiaquine dihydrochloride, and cycloguanil and in methanol for doxycycline, quinine, artemether, and pyrimethamine. Two-fold serial dilutions were prepared in sterile distilled water. Final concentrations, ranging from 0.5 to 1,000 μM for doxycycline, 25 to 3,200 nM for chloroquine, 50 to 2,200 nM for quinine, 3.1 to 400 nM for amodiaquine, 0.4 to 100 nM for artemether, 50 to 40,000 nM for pyrimethamine, and 10 to 20,000 nM for cycloguanil, were distributed in triplicate into Falcon 96-well flat-bottomed plates (Becton Dickinson, 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, 200 μl well of the suspension of parasitized erythrocytes was distributed in 96-well plates precoated with antimalarial agents. Parasite growth was assessed by adding 1 μCi of [3H]-hypoxanthine with a specific activity of 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 19% O₂, 6% CO₂, and 84% N₂, and a humidity of 95%. Immediately after incubation, the plates were frozen, then thawed to lyse erythrocytes. The contents of each well were reduced to 0.25%. Experiments were conducted as described previously and the plates were harvested after 48 hr in 48-hr incubations. In 96-hr experiments, after 48 hr 10 μl of medium containing [3H]-hypoxanthine were added. After an additional 48 hr, the microtiter plates were harvested as described above.

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. Data were analyzed after logarithmic transformation and 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. Isolates were considered chloroquine-resistant if the IC₅₀ was greater than 100 nM. Assessment of doxycycline cross-resistance with the other antimalarials (chloroquine, quinine, amodiaquine, artemether, pyrimethamine, and cycloguanil) was estimated by the Pearson correlation coefficient (r) and coefficient of determination (r²). The significance level was calculated using the correction of Fisher, namely Snr%n, where n is the number of comparisons. For 6 tests carried out simultaneously, the significance level applied to each test was therefore P = 0.05/6 = 0.0083.

**RESULTS**

The following proportions of isolates were successfully cultured for each drug tested: 71 of 104 for doxycycline, artemether, and pyrimethamine, 70 of 104 for chloroquine, 69 of 104 for cycloguanil, and 67 of 104 for quinine and amodiaquine. Average parameter estimates for the 7 compounds against all isolates are given in Table 1.

The IC₅₀ values for doxycycline were in a range from 0.7 to 108.0 nM and the geometric mean IC₅₀ for the 71 isolates was 11.3 μM (95% CI = 9.5–13.4 μM). The activity of doxycycline did not differ significantly (P = 0.0858) between the chloroquine-susceptible isolates and the chloroquine-resistant isolates (Table 2).

There was no significant correlation between the response to doxycycline and that to artemether, chloroquine, quinine, amodiaquine, pyrimethamine, and cycloguanil (Table 3).

In the 96-hr incubations, potency was 4-5-fold increased (P < 0.001) (Table 4). The in vitro responses of doxycycline in the 48-hr and 96-hr incubations were significantly correlated (r = 0.605, P = 0.0047).

**TABLE 1**

<table>
<thead>
<tr>
<th>Drug pair</th>
<th>No.</th>
<th>r</th>
<th>r²</th>
<th>P</th>
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</thead>
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<tr>
<td>Doxycycline-Chloroquine</td>
<td>70</td>
<td>+0.088</td>
<td>0.008</td>
<td>0.4681</td>
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<tr>
<td>Doxycycline-Quinine</td>
<td>67</td>
<td>+0.035</td>
<td>0.001</td>
<td>0.7795</td>
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<tr>
<td>Doxycycline-Amodiaquine</td>
<td>67</td>
<td>+0.050</td>
<td>0.002</td>
<td>0.6898</td>
</tr>
<tr>
<td>Doxycycline-Artemether</td>
<td>71</td>
<td>+0.236</td>
<td>0.065</td>
<td>0.0313</td>
</tr>
<tr>
<td>Doxycycline-Pyrimethamine</td>
<td>69</td>
<td>+0.163</td>
<td>0.026</td>
<td>0.1818</td>
</tr>
<tr>
<td>Doxycycline-Cycloguanil</td>
<td>68</td>
<td>+0.200</td>
<td>0.010</td>
<td>0.1014</td>
</tr>
</tbody>
</table>

* r = Pearson correlation coefficient, r² = coefficient of determination. The significance level was calculated using the correction of Fisher, namely Snr%n, where n is the number of comparisons. For 6 tests carried out simultaneously, the significance level applied to each test was therefore P = 0.05/6 = 0.0083.
In vitro activity of doxycycline on 20 Plasmodium falciparum isolates after an exposure of 48 hr and 96 hr

<table>
<thead>
<tr>
<th>Duration of exposure</th>
<th>Mean IC₅₀</th>
<th>95% confidence intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>48 hr</td>
<td>20.9 μM</td>
<td>14.2-30.7 μM</td>
</tr>
<tr>
<td>96 hr</td>
<td>5.6 μM</td>
<td>3.3-9.6 μM</td>
</tr>
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</table>

*D Values are the geometric mean 50% inhibitory concentrations (IC₅₀).

**DISCUSSION**

Doxycycline was equally potent in vitro against chloroquine-resistant and chloroquine-susceptible isolates. However, doxycycline showed similar activity against all the isolates and showed an IC₅₀ 100-fold greater than those of chloroquine and quinine. Nevertheless, this moderate in vitro activity increases after an exposure of 96 hr. Divo and others demonstrated on clones that potency was increased by prolonged exposure. In addition, antibiotics that inhibit protein synthesis on 70S ribosomes showed marked dependence on O₂ exposure in vitro. The IC₅₀ of 96 hr in high oxygen (15%) and low oxygen (1%) were about the same and 10 times lower than those observed at 48 hr. In the present study, experiments were conducted in 10% O₂. The use of in vitro different criteria such as O₂ tension or time of exposure involves difficulties regarding comparison of data of various studies. The geometric mean IC₅₀ value for doxycycline for the 71 isolates was 11.3 μM. In previous studies of Thai isolates and Cambodian and African strains, the geometric mean IC₅₀ values were 35.4 μM for tetracycline and 5.10 μM and 4.3 μM, respectively, for doxycycline. The in vitro assay system used in these studies differs from that of our work (17% O₂ and 5% O₂). However, doxycycline seems to be more active in vitro than tetracycline.

In addition, doxycycline did not show cross-resistance with arteether, chloroquine, quinine, amodiaquine, pyrimethamine, and cycloguanil. However, molecules considered as promising antimalarial drugs (pyronaridine and artemether) differ from that of our work (17% O₂ and 5% O₂). Nevertheless, doxycycline showed similar activity against all the isolates after an exposure of 48 hr and 96 hr.

The lack of correlation between doxycycline and amino-4-quinolines or aminoalcohols is likely due to the difference in the targets of the drugs. Tetracycline acts on the mitochondrial24 and depresses the activity of dihydroorotate dehydrogenase of the pyrimidine pathway in P. falciparum, presumably due to inhibition of enzyme protein synthesis. Doxycycline reduces levels of malaria nucleoside 5'-triphosphates and doxycycline nucleoside 5'-triphosphates27 and has shown inhibitory effects against pre-erythrocytic stages.13,28

No large studies on doxycycline safety and toxicity have been reported. The severity and disability of side effects have not been well defined. Nevertheless, minor side effects have been reported in doxycycline users. The efficacy of doxycycline alone or in combination with mefloquine, atovaquone, or arteether in prevention or treatment of falciparum malaria has been confirmed in a small number of studies on nonimmune soldiers and local populations.

The in vitro activity of doxycycline against intra-erythrocytic stages of multidrug-resistant P. falciparum, its action against the pre-erythrocytic forms, the lack of correlation between the responses in vitro of doxycycline and the other antimalarial drugs, its original potential site of action, and its efficacy in vivo are factors that favor the use of doxycycline as an antimalarial drug.

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REFERENCES

11. McCollin AA, McHardy N, 1984. Evaluation of a range of anti-


