

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 (Diello 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 antimalarial 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.^{1,2} Failures of antimalarial prophylaxis with chloroquine, the combination of chloroquine and proguanil,³ and mefloquine,^{4,5} and clinical failures with halofantrine⁶ and quinine^{7,8} have been observed. This has led to a search for an effective alternative antimalarial drug with minimal side effects. This emergence and spreading of parasite resistance to currently used antimalarial drugs indicates that novel compounds need to be discovered and developed by identification of novel chemotherapeutic targets.⁹

Thirty years ago, tetracyclines were found to have antimalarial activity.¹⁰ Experimental observations obtained *in vitro*^{11,12} and in clinical studies¹³ showed antimalarial activity of tetracycline and its derivatives. Tetracycline depresses the activity of dihydroorotate dehydrogenase of the pyrimidine pathway in *P. falciparum*,¹⁴ presumably due to inhibition of enzyme protein synthesis. Daily doxycycline has been shown to be an effective causal chemoprophylactic in Thailand,¹⁵ Indonesia,¹⁶ and Kenya.¹⁷ Doxycycline is currently one of the recommended chemoprophylactic regimens for travelers or soldiers visiting Southeast Asia.¹⁸ However, few data are available on its activity against isolates and on the mechanism of antimalarial action. One study assessed the activity of doxycycline against 12 strains or clones and 20 isolates.¹⁹

The aim of the present study was to determine the *in vitro* activity of doxycycline on 71 *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 Diello and Ndiop (280 km southeast of Dakar) in Fatick region of Senegal. Patients from Diello and Ndiop were recruited at home by daily active case detection during a longitudinal study of the mechanisms of protective immunity to malaria.^{20,21} Venous blood was collected into Vacutainer® ACD tubes (Becton Dickinson, Rutherford, NJ) before treatment and transported at 4°C to our laboratory in Marseille. Informed oral consent was obtained from the patients and or their parents before collection of blood. All programs were reviewed and approved by the Conseil de Perfectionnement de l'Institut Pasteur de Dakar. Thin blood smears were stained 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 sensitivity. 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 SP823 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 diphosphate, quinine hydrochloride, amodiaquine dihydrochloride, and pyrimethamine were obtained from Sigma (St. Louis, MO), artemether was obtained from Rhône Poulenc Rorer Doma (Antony, France), and cycloguanil was obtained from Zeneca Pharma (Reims, France). Stock solutions were prepared in sterile distilled water for chloroquine diphosphate,



TABLE 1

In vitro activities of the 7 drugs tested against Gabonese wild isolates of *Plasmodium falciparum**

Drug	Isolate no.	Mean IC ₅₀	95% confidence intervals
Doxycycline	71	11.3 μM	9.5–13.4 μM
Chloroquine	70	76 nM	58–99 nM
Quinine	67	150 nM	128–175 nM
Amodiaquine	67	9.3 nM	8.3–10.4 nM
Artemether	71	3.1 nM	2.5–3.8 nM
Pyrimethamine	71	518 nM	276–969 nM
Cycloguanil	69	176 nM	105–298 nM

* Values are the geometric mean 50% inhibitory concentrations (IC₅₀s).

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 3,200 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 of the suspension of parasitized erythrocytes was distributed in 96-well plates pre-dosed 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, Dreiech, Germany) to each well. Plates were incubated for 42 hr at 37°C in an atmosphere of 10% 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 collected on standard filter microplates (Unifilter[®] GF/B; Packard Instrument Company, Meriden, CT) and washed using a cell harvester (FilterMat[®] Cell Harvester; Packard Instrument Company). Filter microplates were dried and 25 μl of scintillation cocktail (Microscint[®]; Packard Instrument Company) was placed in each well. Radioactivity incorporated by the parasites was measured using a scintillation counter (Top Count[®]; Packard Instrument Company).

In 48-hr and 96-hr experiments, parasitemias were initially reduced to 0.25%. Experiments were conducted as described previously and the plates were harvested after 48 hr in 48-hr experiments. In 96-hr experiments, after 48 hr 10 μl of medium containing ³H-hypoxanthine were added. After an additional 48 hr, the microliter 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

TABLE 2

In vitro susceptibility of Senegalese isolates of *Plasmodium falciparum* to doxycycline and chloroquine*

Drug	Chloroquine-susceptible isolates (n = 49)		Chloroquine-resistant isolates (n = 21)		P
	IC ₅₀ (nM) means	95% confidence limits	IC ₅₀ (nM) means	95% confidence limits	
Chloroquine	40.2	34.0–47.5	339.6	274.7–419.9	
Doxycycline	12.3	10.1–14.9	8.9	6.5–12.3	0.0858

* Values are the geometric mean 50% inhibitory concentrations (IC₅₀s). The threshold IC₅₀ value for resistance to chloroquine is > 100 nM.

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 (*r*²). The significance level was calculated using the correction of Fisher,²² namely 5/*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 μM 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 3

Correlation of *in vitro* responses of Senegalese isolates of *Plasmodium falciparum* to doxycycline, chloroquine, quinine, amodiaquine, artemether, pyrimethamine, and cycloguanil*

Drug pair		No.	<i>r</i>	<i>r</i> ²	<i>P</i>
Doxycycline	Chloroquine	70	+ 0.088	0.008	0.4681
Doxycycline	Quinine	67	+ 0.035	0.001	0.7795
Doxycycline	Amodiaquine	67	+ 0.050	0.002	0.6898
Doxycycline	Artemether	71	+ 0.256	0.065	0.0313
Doxycycline	Pyrimethamine	69	+ 0.163	0.026	0.1818
Doxycycline	Cycloguanil	68	+ 0.200	0.040	0.1014

* *r* = Pearson correlation coefficient; *r*² = coefficient of determination. The significance level was calculated using the correction of Fisher, namely 5/*n* percent, 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.

TABLE 4

In vitro activity of doxycycline on 20 *Plasmodium** *falciparum* isolates after an exposure of 48 hr and 96 hr

Duration of exposure	Mean IC ₅₀	95% confidence intervals
48 hr	20.9 µM	14.2–30.7 µM
96 hr	5.6 µM	3.3–9.6 µM

* Values are the geometric mean 50% inhibitory concentrations (IC₅₀s).

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.¹² In addition, antibiotics that inhibit protein synthesis on 70S ribosomes showed marked dependence on O₂ exposure *in vitro*. The IC₅₀s at 96 hr in high oxygen (15%) and low oxygen (1%) were generally about 100 times and 10 times lower than those observed at 48-hr.¹² 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 isolates²³ and Cambodian and African strains,¹⁹ 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 artemether, chloroquine, quinine, amodiaquine, pyrimethamine, and cycloguanil. However, molecules considered as promising antimalarial drugs (pyronaridine and artemisinin derivatives) showed *in vitro* cross-resistance with chloroquine, quinine, amodiaquine, and halofantrine.^{24,25} The relationship between *in vitro* and *in vivo* resistance depends not only on this but also on the level of resistance and the coefficients of correlation (*r*) and determination (*r*²). A positive correlation between the responses of 2 antimalarial drugs suggests *in vitro* cross-resistance but does not necessarily imply *in vivo* cross-resistance.

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 mitochondrion²⁶ and depresses the activity of dihydroorotate dehydrogenase of the pyrimidine pathway in *P. falciparum*,¹⁴ presumably due to inhibition of enzyme protein synthesis. Doxycycline reduces levels of malaria nucleoside 5'-triphosphates and deoxynucleoside 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.²⁹ The efficacy of doxycycline alone^{29–31} or in combination with mefloquine,³²

atovaquone,³³ or artemether³⁴ 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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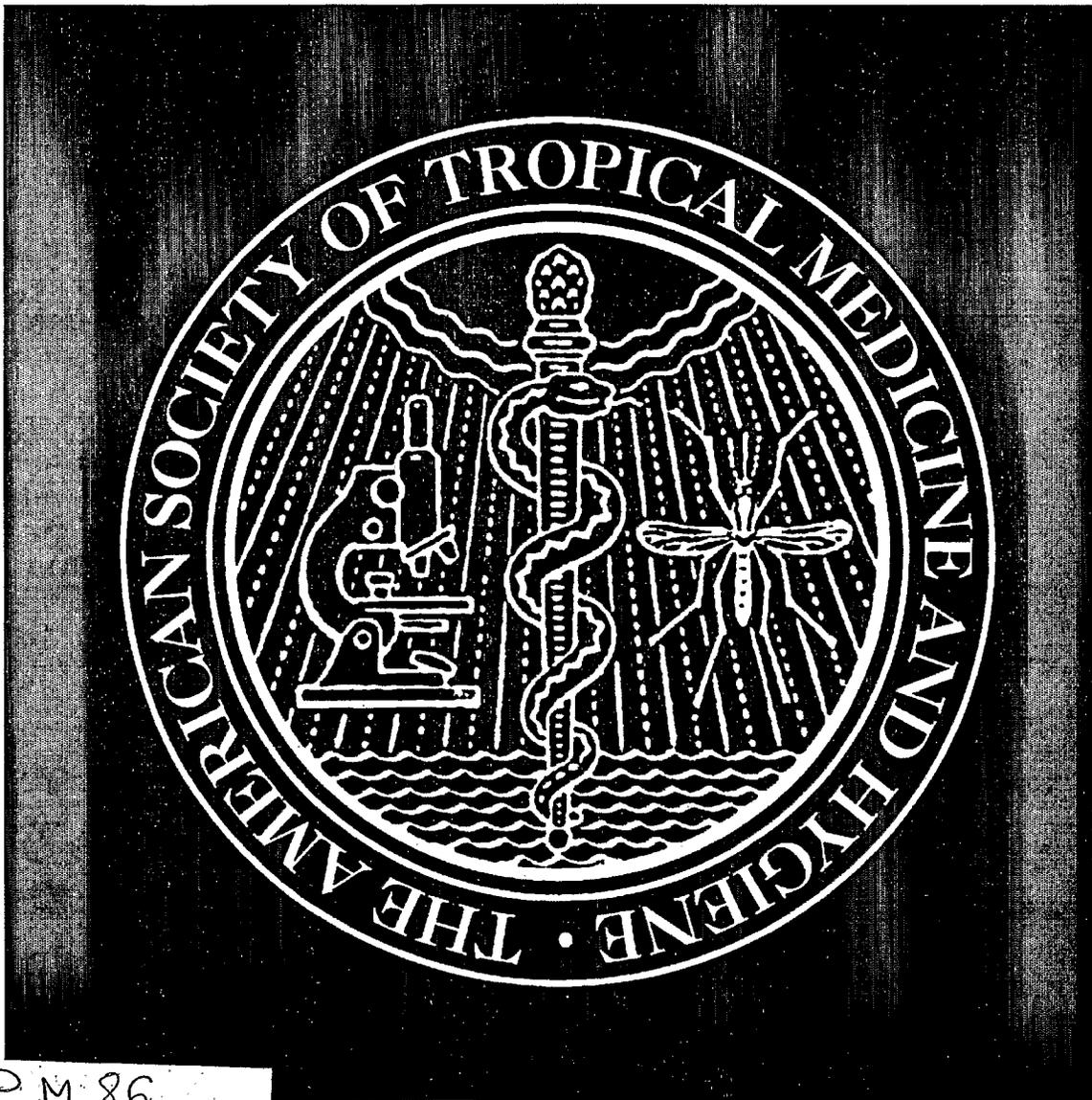
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