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In Vitro Activities of Antibiotics against *Plasmodium falciparum* Are Inhibited by Iron

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The in vitro activities of cyclines (tetracycline, doxycycline, minocycline, oxytetracycline, and rolitetracycline), macrolides (erythromycin, spiramycin, roxithromycin, and lincomycin), quinolones (norfloxacin and ofloxacin), rifampin, thiamphenicol, tobramycin, metronidazole, vancomycin, phosphomycin, and cephalosporins (cephalexin, cefaclor, cefamandole, cefuroxime, ceftriazone, cefotaxime, and cefoxitin) were evaluated on *Plasmodium falciparum* clones, using an isotopic, micro-drug susceptibility test. Only tetracyclines, macrolides, quinolones, and rifampin demonstrated in vitro activity against *P. falciparum*, which increased after a prolonged exposure (96 or 144 h). In the presence of iron ($FeCl_3$), only the activities of tetracyclines and norfloxacin were decreased. Their in vitro activity against intraerythrocytic stages of multidrug-resistant *P. falciparum* and their efficacy in vivo favor the use of antibiotics as antimalarial drugs. However, due to their slow antimalarial action and to the fact that they act better after prolonged contact, they probably need to be administered in conjunction with a rapidly acting antimalarial drug, such as a short course of chloroquine or quinine.

Two of the current options to reduce the morbidity and mortality of malaria are chemoprophylaxis and chemotherapy. During the past 20 years, strains of *Plasmodium falciparum* have become resistant to chloroquine and other antimalarial drugs (49, 53). The search for effective alternative antimalarial drugs with minimal side effects has stimulated work on the identification of novel chemotherapeutic targets (36).

In the past, the use of antibiotics has controlled antimalarial drug-resistant strains. Thirty years ago, tetracyclines were found to have antimalarial activity (43). Experimental observations obtained in vitro (3, 40) and in clinical studies (4, 16) proved the antimalarial activity of doxycycline. Macrolide antibiotics, such as erythromycin and azithromycin, also have both in vitro activity against *P. falciparum* (11, 13) and clinical activity (42, 48). Similarly, fluoroquinolones were also proven to have antimalarial activity in vitro (19, 56) and in vivo (50).

In all living organisms, iron is an essential growth element (50). This metallic compound is needed for the catalysis of DNA synthesis and for a variety of enzymes concerned in electron transport and energy metabolism. Iron chelation has been considered as a suitable therapy for various infectious diseases, including malaria (20). It is required for a large number of the parasite enzyme systems necessary for growth and proliferation, including dihydroorotate dehydrogenase for the synthesis of pyrimidines, phosphoenolpyruvate carboxykinase in CO_2 fixation, cytochrome oxidase in the mitochondrial electron transport system, and superoxide dismutase in oxygen radical catabolism. Also, the iron-dependent enzyme ribonucleotide reductase, a rate-limiting enzyme in DNA synthesis, has been considered as a potential site of action for iron chelators (2, 39). Some antibiotics could repress the activity of enzymes, which are iron-dependent enzymes, such as tetracycline and the dihydroorotate dehydrogenase (23, 41).

The aim of the present study was (i) to determine the in vitro activity of 24 antibiotics from 11 different classes on chloroquine-resistant *P. falciparum* strains after 48, 96, and 144 h of exposure and (ii) to verify the iron binding capacity of the antibiotics with an in vitro activity against *P. falciparum* (tetracyclines, macrolides, quinolones, and rifampin).

MATERIALS AND METHODS

Strains of *P. falciparum*. One chloroquine-susceptible clone, 3D7 (Africa), and four chloroquine resistant clones or strains, W2 (Indochina), Palo Alto (Uganda), FCR3 (Gambia), and Bres1 (Brazil), were maintained in culture. When required for drug assays, cultures were synchronized by sorbitol lysis (25). Susceptibility to antibiotics was determined after suspension in RPMI 1640 medium (Life Technologies, Paisley, United Kingdom) supplemented with 10% human serum (pooled from different A^+ or AB sera from nonimmune donors who did not live in the area of malaria endemicity) and buffered with 25 mM HEPES and 25 mM $NaHCO_3$ (hematocrit of 1.5%, parasitemia of 0.5%).

Drugs. All the antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.). Stock solutions were prepared (i) in methanol for tetracycline, doxycycline, minocycline, oxytetracycline, erythromycin, spiramycin, roxithromycin, lincomycin, thiamphenicol, cephamandole, cephalexin, cefuroxime, ceftriazone, cefotaxime, cefoxitin, metronidazole, phosphomycin, and rifampin or (ii) in dimethyl sulfoxide for ofloxacin, norfloxacin, rolitetracycline, tobramycin, vancomycin, and cefaclor. Twofold serial dilutions were prepared in sterile distilled water or RPMI for all these drugs. Final concentrations were distributed in triplicate into Falcon 96-well flat-bottom plates (Becton Dickinson, Franklin Lakes, N.J.), which were dried.

In vitro assays. The isotopic micro drug susceptibility test used in this study was described previously (38). For in vitro isotopic microtests, 200 μl of the suspension of parasitized erythrocytes (parasitemia of 0.5% for the 48-h test, 0.2% for the 96-h test, and 0.1% for the 144-h test) per well was distributed in 96-well plates predosed with antimalarial agents. Parasite growth was assessed by adding 1 μCi of [^{3}H]hypoxanthine with a specific activity of 14.1 Ci/mmol (NEN Products, Dreiech, Germany) to each well at 0 h for the 48-h exposure test, at 48 h for the 96-h exposure test, and at 96 h for the 144-h exposure test. Plates were incubated for 48, 96, or 144 h at 37°C in an atmosphere of 10% O_2, 6% CO_2, and 84% N_2 and 95% relative humidity.

The 50% inhibitory concentration (IC_{50}), i.e., the drug concentration corre-

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TABLE 1. In vitro activity of tetracyclines, macrolides, quinolones, and rifampin against a chloroquine-susceptible clone, 3D7, and chloroquine-resistant strains, W2, Palo Alto, FCR3, and Bres1

Antibiotic	Mean IC_{50} (μM) (95% confidence interval)[a] against:				
	3D7	W2	FCR3	Bres1	Palo Alto
Doxycycline	9.7 (8.1–11.5)	7.7 (7.1–8.4)	12.4 (10.9–14.0)	14.9 (11.5–19.3)	11.2 (10.0–12.5)
Tetracycline	42.7 (36.3–50.2)	33.8 (26.4–43.3)	62.2 (43.8–88.5)	88.9 (60.3–131.1)	88.1 (66.9–116.0)
Minocycline	10.2 (8.5–12.3)	16.0 (11.8–21.6)	15.6 (12.4–18.8)	17.0 (14.3–20.3)	12.3 (9.6–15.8)
Oxytetracycline	34.4 (19.5–60.7)	46.0 (44.2–47.9)	54.5 (50.6–58.4)	67.5 (63.3–71.9)	47.9 (44.1–51.9)
Rolitetracycline	208.0 (172.8–250.2)	45.5 (35.2–58.8)	155.9 (137.8–174.0)	117.5 (110.3–125.2)	203.2 (168.9–244.5)
Norfloxacin	55.3 (39.1–78.3)	74.6 (70.7–78.8)	54.1 (38.5–75.9)	58.6 (40.7–84.5)	75.0 (66.1–85.1)
Ofloxacin	152.1 (109.4–211.4)	112.5 (105.1–120.3)	113.8 (103.0–125.6)	158.1 (152.5–163.9)	195.0 (152.8–248.8)
Rifampin	3.2 (2.8–3.7)	1.3 (1.0–1.5)	1.7 (1.5–2.9)	1.9 (1.4–2.6)	2.1 (1.7–2.7)
Erythromycin	>250	39.4 (27.9–55.5)	18.0 (14.9–21.6)	24.3 (21.9–26.9)	17.5 (12.8–24.0)
Spiramycin	69.0[b] (53.1–89.7)	20.9[b] (18.6–23.5)	19.0[b] (15.9–22.7)	13.7[b] (9.2–20.4)	20.8[b] (17.0–25.5)
Roxithromycin	>250	18.6 (14.6–23.6)	12.8 (8.1–20.2)	13.3 (8.2–21.7)	6.8 (4.7–9.9)

[a] Values are the geometric mean IC_{50} of 5 to 10 assays.
[b] Units per milliliter.

sponding to 50% of the uptake of [^{3}H]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 IC_{50} with 95% confidence intervals.

Iron-induced inhibition of antibiotic activity. A range of concentrations of antibacterial drugs, prepared in RPMI, were preincubated with $FeCl_3$ (Sigma Chemical Co.) at 500, 750, and 1,000 μM (concentrations in final test) in RPMI for 3 h. Positive controls were determined with an iron-chelating agent, desferrioxamine (Sigma). For the in vitro microtest, 50 μl of the complex and 150 μl of a suspension of parasitized erythrocytes (parasitemia of 0.5% and hematocrit of 1.5% in final) were distributed in 96-well plates. The procedure was as described in above for in vitro assays.

RESULTS

Table 1 gives the IC_{50}s of the different tetracyclines, macrolides, quinolones, and rifampin shown to have antimalarial activity (<250 μM) against the clone W2. The in vitro growth inhibition of these antibiotics was also tested on four other *P. falciparum* strains: one chloroquine-susceptible strain (3D7) and three chloroquine-resistant strains (FCR3, Palo Alto, and Bres1). Lincomycin (macrolide), tobramycin (aminoglycoside), thiamphenicol (chloramphenicol derivative), metronidazole (imidazole derivative), vancomycin and phosphomycin (unclassable antibiotics), cephalexin and cefaclor (narrow-spectrum cephalosporins), cefamandole and cefuroxime (extended-spectrum cephalosporins), ceftriazone and cefotaxime (broad-spectrum cephalosporins), and cefoxitin (cephamycin) had IC_{50}s up to 800 μM against the chloroquine-resistant W2 clone.

As shown in Table 2, the antimalarial activity of doxycycline, tetracycline, minocycline, oxytetracycline, rolitetracycline, erythromycin, spiramycin, roxythromycin, norfloxacin, ofloxacin, and rifampin was increased in vitro after prolonged exposure.

Table 3 shows that the activities of doxycycline, tetracycline, minocycline, oxytetracycline, rolitetracycline, norfloxacin, and desferrioxamine were inhibited in the presence of iron ($FeCl_3$).

DISCUSSION

Thirty years ago, tetracycline and its derivatives were found to have antimalarial activity (43), as demonstrated by in vitro (6, 30) and clinical (54) studies. The IC_{50} for tetracyclines in our experiments was close to those reported by other authors: 39 μM for oxytetracycline (6), 32 μM for tetracycline (55), and 11 μM for doxycycline (40). Doxycycline and minocycline were the most potent tetracyclines. Enhanced potency may be due to their increased lipophilicity, facilitating their entry into cells (45). Tetracyclines tended to act slowly against malaria parasites, with an increased activity after prolonged exposure (96 and 144 h). In comparison, standard antimalarial drugs, such as the fast-acting amino-4-quinoleines or amino alcohols, do not vary in activity over time of contact (21). Because of the slow activity of tetracyclines, they would probably be administered in a regimen that also included a rapidly acting drug. Tetracyclines act on malaria parasite mitochondria, which, similar to eucaryotes, contains 70S ribosomes. Tetracycline also inhibits protein synthesis on 80S ribosomes in broken-cell preparations (8). However, a low accumulation of tetracycline is observed, which is consistent with a mitochondrial effect (12). If tetracycline had attained high intraparasitic concentrations, 80S ribosomal inhibition could play a role in the antimalarial effect. Tetracycline acts on mitochondria (22) and depresses the activity of dihydroorotate dehydrogenase, an iron-dependent enzyme of the pyrimidine pathway in *P. falciparum* (23, 41), presumably due to the inhibition of enzyme protein synthesis. Doxycycline reduces the levels of malaria nucleoside 5′-triphosphates and deoxynucleoside 5′-triphosphates (58) and

TABLE 2. In vitro activity of tetracyclines, macrolides, quinolones, and rifampin against the chloroquine-resistant clone W2 after 48, 96, and 144 h of contact exposure

Antibiotic	Mean IC_{50} (μM) (95% confidence interval)[a] after		
	48 h	96 h	144 h
Doxycycline	7.7 (7.1–8.4)	1.28 (1.25–1.3)	0.5 (0.3–0.8)
Tetracycline	33.8 (26.4–43.3)	4.1 (2.3–7.4)	0.8 (0.4–1.4)
Minocycline	16.0 (11.8–21.6)	3.13 (3.11–3.14)	0.3 (0.2–0.3)
Oxytetracycline	46.0 (44.2–47.9)	16.0 (13.1–19.6)	1.5 (0.9–2.4)
Rolitetracycline	45.5 (35.2–58.8)	10.38 (10.33–10.42)	3.7 (1.9–7.5)
Norfloxacin	74.6 (70.7–78.8)	27.9 (14.7–52.9)	5.8 (4.4–7.6)
Ofloxacin	112.5 (105.1–120.3)	99.8 (95.6–106.3)	19.3 (12.0–31.2)
Rifampin	1.3 (1.0–1.5)	0.20 (0.18–0.22)	0.09 (0.08–0.11)
Erythromycin	39.4 (27.9–55.5)	6.0 (4.1–8.8)	1.9 (1.0–3.7)
Spiramycin	20.9[b] (18.6–23.5)	5.0[b] (1.9–13.3)	3.1[b] (2.9–3.3)
Roxithromycin	18.6 (14.6–23.6)	4.8 (4.6–4.9)	1.9 (1.3–2.8)

[a] Values are the geometric mean IC_{50} of three assays.
[b] Units per milliliter.

TABLE 3. In vitro activity of tetracyclines, macrolides, quinolones, rifampin, and desferrioxamine in combination with $FeCl_3$ against the chloroquine-resistant clone W2 after 48 h of contact exposure

Molecule	Mean IC_{50} (μM) (95% confidence intervals)[a] in combination with $FeCl_3$ at:			
	0 μM	500 μM	750 μM	1,000 μM
Desferrioxamine	11.1 (9.6–12.9)	42.2 (37.2–47.9)	>100	>100
Doxycycline	8.0 (7.8–8.2)	10.1 (9.7–10.5)	10.1 (9.4–10.9)	10.1 (9.4–10.8)
Tetracycline	37.2 (35.6–38.9)	46.2 (42.2–50.6)	44.4 (40.4–48.8)	48.5 (44.1–55.3)
Minocycline	19.6 (16.0–24.0)	30.2 (26.4–34.6)	>50	>50
Oxytetracycline	43.9 (42.7–45.1)	76.0 (71.7–80.6)	80.7 (75.4–86.4)	87.1 (79.6–95.3)
Rolitetracycline	52.0 (47.1–57.5)	75.2 (71.6–79.0)	83.0 (78.7–87.7)	82.1 (78.8–85.5)
Norfloxacin	76.9 (71.6–82.7)	96.2 (90.3–102.4)	108 (99–118)	111 (105–119)
Ofloxacin	107 (103–113)	106 (98–115)	97 (90–104)	94.4 (90.2–98.7)
Rifampin	1.4 (1.3–1.5)	1.5 (1.4–1.7)	1.5 (1.4–1.6)	1.4 (1.3–1.6)
Erythromycin	40.7 (34.2–48.6)	39.8 (33.4–47.5)	37.7 (30.5–46.6)	40.5 (37.2–44.2)
Spiramycin	21.8[b] (20.3–23.5)	21.3[b] (19.7–23.1)	22.0[b] (20.7–23.3)	20.6[b] (18.4–23.1)
Roxithromycin	20.9 (16.4–26.5)	22.6 (19.7–23.1)	23.6 (20.8–26.7)	20.4 (18.4–23.1)

[a] Values are the geometric mean IC_{50} of three assays.
[b] Units per milliliter.

has shown inhibitory effects against preerythrocytic stages (28). Tetracyclines could also act on the dihydroorotate dehydrogenase enzyme by chelating its iron center. The efficacy of doxycycline alone (17, 52) or in combination with mefloquine (27) or atovaquone (26) in prevention or treatment of falciparum malaria has been confirmed.

The IC_{50}s of norfloxacin, which were similar to those reported by other authors (in 48-h drug exposure tests) (19, 32), and ofloxacin in our experiments confirm that quinolones are potent against *P. falciparum*. Norfloxacin is approximately twice as potent in vitro than is ofloxacin and is effective against both chloroquine-susceptible and chloroquine-resistant parasites, more so after prolonged exposure (96 or 144 h) (56). The mode of action of quinolones against *Plasmodium* species is still unknown. However, these agents act against bacteria through inhibition of DNA gyrase, a prokaryotic type II topoisomerase (29). Nevertheless, the relatively slow antimalarial action of fluoroquinolones limits their clinical application in treating falciparum malaria. Consequently, quinolones could be used as alternatives as part of a combination therapy.

Macrolides, such as erythromycin, were shown to be active against *P. falciparum* (21, 44). The IC_{50} of erythromycin in our experiments was close to those reported by other authors, 68 μM (13). Erythromycin is active against the chloroquine-resistant strains (IC_{50}, 17 to 40 μM) but seems to be inactive against the chloroquine-susceptible clone (IC_{50}, >250 μM). In contrast, erythromycin is inactive against chloroquine- and quinine-resistant malaria in Thailand (37). Azithromycin, a new macrolide antibiotic with a longer half-life and a better bioavailability, has microbiological and pharmacokinetic advantages over erythromycin (7). The antimalarial activity of azithromycin has been demonstrated against *P. falciparum* (13), *P. yoelii* (1, 14), and *P. cynomolgi* (42) and in clinical studies (24). Malaria could be a future indication for macrolides (47), although they would be better used as part of a combination therapy (35). In our experiments, macrolide activities were increased in vitro during prolonged exposure, the same as for azithromycin (57).

Rifampin was the most potent of the antibiotics tested in this study. The IC_{50} of rifampin in our experiments was close to those reported by other authors: greater than 2 μM (46). A putative prokaryote-like RNA polymerase in *P. falciparum* has been suggested as one possible target of rifampin (10). A fixed combination of rifampin, co-trimoxazole, and isoniazid was found to be highly effective for the treatment of malaria (9, 15). Rifampin was found to be more active after prolonged exposure, in contrast to the results of Divo et al. (6).

Only the activities of the tetracyclines and norfloxacin were inhibited by iron. Inhibition of activity was more pronounced for minocycline and oxytetracycline. The ratio of the antibiotic IC_{50} with $FeCl_3$ to that of the antibiotic IC_{50} without $FeCl_3$ was greater than 2 for minocycline combined with 750 μM $FeCl_3$ and for oxytetracycline combined with 1,000 μM $FeCl_3$. The IC_{50} of desferrioxamine was 4- to >10-fold higher when combined with $FeCl_3$. The reversal of the inhibition of growth obtained by doxycycline, tetracycline, rolitetracycline, and norfloxacin combined with $FeCl_3$ was weaker than that obtained with minocycline, oxytetracycline, and desferrioxamine. With the latter antibiotics, the IC_{50} was not influenced by $FeCl_3$ at 500 to 1,000 μM. Using a siderophore colorimetric assay, doxycycline and minocycline were found to have a strong iron-chelating activity, whereas that of tetracycline was less pronounced (18). However, Grenier et al. (18) found that the interaction between iron and doxycycline or tetracycline appears not to strongly affect the antibacterial activity. Miles and Maskell concluded that the complexing of tetracycline with iron in vivo did not affect the efficacy of tetracycline but, rather, diminished the iron-induced enhancement of the infection (33, 34). In our study, we showed that $FeCl_3$ decreased in vitro activity of tetracyclines and norfloxacin, like the iron-chelating agent desferrioxamine. In addition, tetracycline can complex with oxovanadium(IV) (5). As a result, the efficacy of tetracyclines used as antimalarial or antibacterial agents could be reduced in patients with iron overload. Also, the combination of tetracyclines with compounds which require iron for their antimalarial activities, such as artemisinin and its derivatives (31), may be unadvisable. Nevertheless, the efficacy of doxycycline combined with artemether in prevention or treatment of multidrug-resistant falciparum malaria has been confirmed, but recrudescence was observed in 47% of patients (35). The cure rate of the artemether-doxycycline regimen was significantly lower than that of the mefloquine-doxycycline regimen

(80 and 96%), respectively (27). The efficacy of tetracyclines may be increased if they were combined with iron chelators.

The present study clearly demonstrated the in vitro activity of antibacterial drugs (tetracyclines, macrolides, quinolones, and rifampin) against *P. falciparum*. Their in vitro activity against intraerythrocytic stages of multidrug-resistant *P. falciparum* and their efficacy in vivo favor the use of antibiotics as antimalarial drugs. However, because of their relatively slow antimalarial action and enhanced activity after prolonged contact, they would be best administered in conjunction with a rapidly acting antimalarial drug, such as a short course of chloroquine or quinine. In addition, we showed that the activities of tetracyclines and norfloxacin may be decreased in the presence of $FeCl_3$, in favor of a combination with iron chelators.

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