EFFECTS OF MINOCYCLINE AGAINST MEFLOQUINE-, CHLOROQUINE- AND PYRIMETHAMINE- RESISTANT PLASMODIUM FALCIPARUM IN VITRO

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Abstract: Tetracyclines are used for prophylaxis of malaria and treatment of drug-resistant falciparum malaria because of their safe drug action. We re-evaluated effects of three tetracyclines against drug-resistant Plasmodium falciparum in vitro. Minocycline was approximately 4 times and twice more potent in inhibiting the in vitro growth of falciparum parasites than tetracycline and doxycycline, respectively. Compared with doxycycline, significant inhibitory effects of minocycline to chloroquine, pyrimethamine and mefloquine resistant P. falciparum strains were affirmed by the present in vitro study. By electron microscopy a number of electron dense vesicles with a single membrane bound were observed in the cytoplasm of minocycline-treated parasites, although no distinct structural alternations of mitochondria was noted. Minocycline may be a better therapeutic drug than doxycycline which is widely accepted as the standard antimalarial tetracycline.

Key words: Plasmodium falciparum, Drug-resistance, Electron microscopy, Tetracyline, Minocycline, Chloroquine, Mefloquine, Pyrimethamine

INTRODUCTION

Chemotherapy is the primary defense against malaria. Therefore, the spread of drug resistant Plasmodium falciparum is a world-wide threat. Development of new antimalarials is one of the major goals of malaria research. However, development and deployment of a new drug is extremely expensive, causing discouragement for pharmaceutical companies to search for new therapeutic agents. This leads to re-evaluation of antimalarial activities of any drugs which have already been accepted for clinical use in patients with various infectious diseases other than malaria. Antibiotics are one of the major sources for such trials. Amongst commonly used antibiotics, a group of tetracyclines has been most widely used for treatment of malaria (Puri and Dutta, 1982). The effect of chlortetracycline against a malaria parasite was first reported by Coatney (Coatney et al., 1949). Since then, tetracyclines have been tried in malaria treatment (Pang et al., 1987; Loaareesuwan et al., 1992; Rieckman et al., 1971). The conventional tetracycline is a short acting agent (plasma half-life: 8.5 hr), while doxycycline and minocycline have relatively long half-lives (plasma half-life: 18-22 hr and 12-16 hr, respectively (Dollery, 1999), permitting better selection of these tetracyclines for malaria treatment than tetracycline. Doxycycline in particular, is accepted as an antimalarial for chemotherapy as well as chemoprophylaxis (Colwell et al., 1972; Shanks et al., 1992). The present study focused on the potency of the antimalarial activity of minocycline to cultured drug-resistant P. falciparum parasites in comparison with tetracycline and doxycycline. We show here the first report that minocycline was effective against mefloquine-resistant falciparum parasites in vitro. The presumed molecular mechanisms of tetracyclines and the reason for the highest potency shown by minocycline are discussed.

MATERIALS AND METHODS

Parasites

Two established strains and two recent clinical isolates of P. falciparum were used in the present study (Table 1). SGE-1 is a drug sensitive strain of Gambian origin, donated...
by Dr. P. Ambroise-Thomas of the University of the Grenoble in 1979, and has been maintained by in vitro culture alternating occasional freezing in liquid nitrogen in our laboratory. Regardless of long-term cultivation, the parasite has maintained its virulence, causing fatal sickness in Aotus monkeys. K-1 is a chloroquine-resistant strain isolated in Thailand, which was donated by the London School of Hygiene and Tropical Medicine in 1984. One isolate, MZG, is a chloroquine-sensitive but mefloquine-resistant parasite isolated from a Japanese patient who developed falciparum malaria after returning from Mozambique in 1998. Another isolate NGG is a moderate chloroquine-resistant and highly pyrimethamine-resistant parasite obtained from a Japanese patient who also developed falciparum malaria after visiting Nigeria in 1997.

Cultivation of P. falciparum parasites and in vitro drug susceptibility test

Culture of falciparum parasites was carried out according to a modified method of Trager and Jensen (Trager and Jensen, 1976), using RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Japan) medium with 10% human serum (RPMI 1640 (+)) and type O human red blood cells. The drug susceptibility test was performed by a semi-micro method, described previously (Bras and Deloron, 1983; Inaba et al., 2001). Parasites were synchronized by D-sorbitol treatment (Lambros and Vanderberg, 1979), and parasitized erythrocytes with a 0.15-0.3% infection rate were adjusted to a 5% packed cell volume in RPMI 1640 (+) at the start of incubation. The test was done using 24-well plates (Falcon, 3047, Becton Dickinson, NJ, USA) and each well contained 500 µl of parasitized erythrocyte suspension and 20 µl of drug solution containing the respective concentrations of each drug. Final drug concentrations of tetracyclines were 0, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100 and 300 µg/ml. The incubator was kept at 37°C continuously with a gas flow mixture composed of 5% O2, 5% CO2 and 90% N2. The medium was changed every day and the cultivation was continued for up to 4 days (Divo et al., 1985; Geary and Jensen, 1983; Inaba et al., 2001). The effects of drugs on the growth of the parasites were expressed by the 50% inhibitory concentrations (IC50), which were calculated by computerized probit analysis.

Drugs used and the sources were as follows: tetracycline hydrochloride and minocycline hydrochloride from Lederle Ltd., Japan; doxycycline from Sigma Chemical Co., USA; chloroquine sulfate from Winthrop Stearns Inc., Manila, Philippines; mefloquine from Roche Diagnostics, Switzerland; pyrimethamine from Wako Pure Chemical Industries, Ltd., Japan.

Electron microscopy

Erythrocytes infected with SGE-1 parasites were treated with tetracycline at 1.0 µg/ml and minocycline at 0.3 µg/ml, approximately at the IC50 values described in the text. Blood samples were taken every 24 hr after drug exposure and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 2 hr (Kawai et al., 1996). The specimens were postfixed in 1% osmium tetroxide for another 2 hr, dehydrated in a graded alcohol series, treated with propylene oxide, and embedded in Epon 812. The resultant blocks were cut with a Porter Blum (Newton, CT) MT-2 ultramicrotome with a Diatome (Bienne, Switzerland) diamond knife. Thin sectioned specimens were mounted on 200-mesh copper grids, stained with uranyl acetate plus lead citrate. The prepared specimens were observed using a transmission electron microscope (Hitachi, H-800, Tokyo, Japan).

RESULTS

Drug resistant profiles of P. falciparum parasites used in the present study

Drug susceptibilities of three P. falciparum parasites were examined by a semi-micro in vitro drug susceptibility test, in comparison with SGE-1 as a standard strain (Table 1). The IC50 of K-1 for chloroquine was 4.4-fold higher than that of the SGE-1 strain. The K-1 strain also showed

<table>
<thead>
<tr>
<th>Strain</th>
<th>Chloroquine (µM)</th>
<th>Mefloquine (nM)</th>
<th>Pyrimethamine (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGE-1</td>
<td>0.086±0.002</td>
<td>13.97±2.64</td>
<td>69.1±7.93</td>
</tr>
<tr>
<td>K-1</td>
<td>0.378±0.019</td>
<td>12.78±3.85</td>
<td>126±10.86</td>
</tr>
<tr>
<td>MZG</td>
<td>0.040±0.001</td>
<td>248.3±50.17</td>
<td>75.8±8.52</td>
</tr>
<tr>
<td>NGG</td>
<td>0.22±0.017</td>
<td>4.88±0.89</td>
<td>2,774±109.4</td>
</tr>
</tbody>
</table>

*Values are mean±SD for three experiments

SGE-1 was used as a standard P. falciparum strain which showed susceptibilities to chloroquine, mefloquine and pyrimethamine. K-1, MZG and NGG strains showed selective resistance to chloroquine, mefloquine and pyrimethamine respectively.
moderate resistance to pyrimethamine. The clinical isolate, MZG of Mozambique origin, showed a 17.8-fold higher IC₅₀ for mefloquine. Another clinical isolate, NGG of Nigerian origin, was highly resistant to pyrimethamine with a 40-fold higher IC₅₀ than that of the SGE-1 strain. These findings confirmed that all strains and isolates of falciparum parasites used in the present study had maintained their stable phenotypes in terms of original drug sensitivities to each antimalarial drug.

Comparison of effects of three tetracyclines on the growth of *P. falciparum* parasites in vitro

We compared the inhibitory effects of tetracycline, doxycycline and minocycline on the standard SGE-1 strain by determining the IC₅₀ values at 24, 48, 72 and 96 hr after drug exposure. The antimalarial activities of all three tetracyclines were enhanced when drug exposure was increased from 24 to 96 hr (Table 2), as reported previously (Divo et al., 1985; Geary and Jensen, 1983). However, the inhibitory effect of minocycline was the most potent at each time point examined (Table 2). The IC₅₀ value for minocycline at 24 hr was 3.90 μg/ml, while those for doxycycline and tetracycline were 10.11 and 17.20 μg/ml, respectively. At 96 hr the IC₅₀ for minocycline (0.24 μg/ml) was 2.1-fold less than that for doxycycline (0.50 μg/ml) and 5.1-fold less for tetracycline (1.23 μg/ml). Overall, the mean IC₅₀ value at different time points for minocycline was approximately 4-fold and 2-fold lower than that for tetracycline and doxycycline.

The efficacy of minocycline was further studied using drug-resistant parasites in comparison with tetracycline. Growth inhibition of parasites was assayed in the presence of tetracycline at 1.0 μg/ml or minocycline at 0.3 μg/ml, approximating the IC₅₀ values, according to the above findings. All falciparum parasites, such as SGE-1, chloroquine-resistant K-1, mefloquine-resistant MZG and pyrimethamine-resistant NGG, showed substantially similar susceptibilities to both tetracycline and minocycline (Table 3). The inhibitory effects of these antibiotics were notable at 72 hr with 20-30% growth inhibition. Eventually, the effect reached to

<table>
<thead>
<tr>
<th>Drug</th>
<th>24 hr</th>
<th>48 hr</th>
<th>72 hr</th>
<th>96 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracycline</td>
<td>17.2±1.52</td>
<td>4.36±0.91</td>
<td>2.26±0.38</td>
<td>1.23±0.24</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>10.11±1.84</td>
<td>2.45±0.14</td>
<td>1.10±0.01</td>
<td>0.50±0.13</td>
</tr>
<tr>
<td>Minocycline</td>
<td>3.90±1.60</td>
<td>2.00±0.25</td>
<td>0.85±0.22</td>
<td>0.24±0.01</td>
</tr>
</tbody>
</table>

By *in vitro* comparative study on tetracyclines on SGE-1 strain, it is remarked that minocycline showed 4.4 times higher anti-malarial activity than that shown by doxycycline at 24 hr test. It is notable that minocycline shows inhibitory effect to the parasites at the early stage of the test.

<table>
<thead>
<tr>
<th>Drug*</th>
<th>strain</th>
<th>% Inhibition of growth at time points †</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hr</td>
<td>48 hr</td>
</tr>
<tr>
<td>MC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SGE-1</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>K-1</td>
<td>-12</td>
<td>16</td>
</tr>
<tr>
<td>MZG</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>NGG</td>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>TC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SGE-1</td>
<td>-9</td>
<td>26</td>
</tr>
<tr>
<td>K-1</td>
<td>-6</td>
<td>2</td>
</tr>
<tr>
<td>MZG</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>NGG</td>
<td>-20</td>
<td>2</td>
</tr>
</tbody>
</table>

* TC, tetracycline at 1.0 μg/ml; MC, minocycline at 0.30 μg/ml.
† Inhibitory effect was determined by comparison of the parasite growth of untreated and drug-treated parasites in triplicate experiments.

By 24 hr test with low dose tetracyclines, no effect was remarked. Percent growth inhibitory values were equally elevated at high levels at 96 hr test both in the standard strain and resistant strains. Still, MC effect> TC effect is also noted.
the maximum at 96 hr, resulting in 40-48% inhibition by tetracycline and 65-72% by minocycline.

**Ultrastructural changes of parasites treated with tetracyclines**

The structural alterations of parasites caused by treatment with tetracycline at 1.0 µg/ml and minocycline at 0.3 µg/ml were examined at 24, 48, 72 and 96 hr after treatment. Distinct differences in the structural changes were not observed between parasites treated with tetracycline and minocycline so far as the present study concerns. Enlargement of the perinuclear cisterna space was noted in the specimen at 24 hr of tetracycline treatment, compared with untreated parasites (Fig. 1A, B and C). A dilatation of cisternae of endoplasmic reticulum was also noted at 24 hr and 48 hr (data not shown). In parasites exposed to tetracyclines for 72 hr, a number of electron dense vesicles with a single membrane bound were observed and the cytoplasmic structure was largely disintegrated (Fig. 1D). However, distinct structural alterations of the organelles, such as mitochondria and plastids, were not noted in the present study.

**DISCUSSION**

The present study showed that in a group of tetracyclines, minocycline was the most potent for suppressing the growth of cultured *P. falciparum*. The IC₅₀ values of tetracycline, doxycycline and minocycline for the *P. falciparum* SGE-1 strain were 1.23, 0.50 and 0.24 µg/ml, respectively. Acute systemic toxicity studies showed that the LD₅₀ value of these drugs, when orally administered, was 2,000-3,000, 1,700 and 1,900-3,600 mg/kg of body weight, respectively (Dollery, 1999; personal communications from Lederle Japan and Pfizer Japan). When simply comparing the ratio of the LD₅₀ versus the IC₅₀, the ratio of minocycline was estimated to be 7,900-15,000, whereas the ratio of tetracycline was 1,600-2,400. This finding indicates that minocycline appears to be 3.3-9.4-fold more potent than tetracycline.

![Figure 1](image-url) Electron micrograph of *P. falciparum* treated with tetracyclines. An early trophozoite (A) and schizont (B) are shown as a control. Early trophozoites were treated with tetracyclines for 24 hr (C) and 72 hr (D). Dilatations of perinuclear cisterna space are indicated by arrows. Electron dense vesicles are indicated by arrow heads. N, nucleus; M, mitochondrion; P, pigment. Bar=0.2 µm
The index of minocycline also exceeds that of doxycycline with a value of 3,400.

Minocycline eventually showed killing activity for mefloquine-resistant *P. falciparum* parasites as well as chloroquine- and pyrimethamine-resistant parasites, although this antibiotics required a longer time for an appearance of the inhibitory effect than other antimalarial drugs. As far as we know, this is the first report on the effect of minocycline against mefloquine-resistant *P. falciparum in vitro*. This finding suggests that minocycline should be further studied in clinical cases as an excellent candidate for chemotherapy of drug-resistant malaria, even though there were some clinical trials for chloroquine-resistant malaria using this antibiotics in the early 1970’s (Colwell et al., 1972; Willerson et al., 1972). Tetracycline has already been used to treat drug-resistant falciparum malaria, generally in combination with quinine (Looareesuwan et al., 1992; Puri and Dutta, 1982; Rieckman et al., 1971; Watt et al., 1992). Our recent experiments showed that a combination of minocycline with artemether and/or chloroquine resulted in an excellent therapeutic effect without showing recrudescence in mice infected with *P. berghei* and chloroquine-resistant *P. chabaudi* (manuscript in preparation).

The rapid anti-parasite action of minocycline in vitro study has also been shown in the present study (Table 2). The inhibitory action shown at the early stage of drug exposure is a key point to evaluate antimalarials. All these results support the use of minocycline as a better tetracycline than doxycycline for the treatment of chloroquine-, pyrimethamine- and mefloquine-resistant falciparum malaria, respectively.

The highly inhibitory effects of minocycline on parasite growth shown in the present study may be attributable to the high lipophilicity of minocycline (Tomas, 1989). Lipophilic features of this antibiotics may permit the high permeability through the plasma membrane, mitochondrial membrane and even plastid membrane. The minocycline may be more efficiently transported from the serum to the parasites in the erythrocytes than the other tetracyclines because of its lipophilic feature. Mitamura et al. (2000) showed that serum albumin (SA) derived fatty acid species in the neutral lipid fraction in the serum plays an crucial role in the parasite growth at the erythrocytic stage. It appears that SA derived fatty acid passes through the erythrocytic membrane by some mechanism. The finding will suggest a probable role of lipid as the carrier for lipophilic minocycline to pass through erythrocytic membrane and eventually reach to the parasites.

The ultrastructural alterations, such as the dilatation of perinuclear space and appearance of electron dense single membrane-bound vesicles were marked in falciparum parasites treated with tetracyclines in the present study. The electron dense vesicles were also observed in late trophozoites and schizonts of *P. falciparum* taken from *Aotus* monkeys treated with pyronaridine (Kawai et al., 1996). It was notable that these alterations in the parasites occurred before no change came out in mitochondria, which is considered as the target of tetracyclines. However, it is difficult, at present, to explain whether the structural changes resulted from the specific action mechanism of tetracyclines. Further investigations into the morphologic changes focusing on the parasite mitochondria and plastids will be of particular importance in future studies.

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**REFERENCES**

of *Plasmodium falciparum* erythrocytic stages in culture. J. Parasitol., 65, 418-420


