Inhibition of mitochondrial and plastid activity of *Plasmodium falciparum* by minocycline

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Abstract We previously reported the superior effect of minocycline against drug-resistant *Plasmodium falciparum* in vitro. Here, we report that RT-PCR for falciparum parasites treated with minocycline revealed reduced levels of RNA transcripts of the mitochondrion-encoded genes such as the COI and Cyb genes, as well as the plastid-encoded RNA polymerase subunit (rpoB/C) gene. However, we detected no apparent effects of the antibiotic on the transcription of merozoite surface antigen and small subunit rRNA genes encoded by the nucleus. In addition, treatment with chloroquine and pyrimethamine showed no substantial reduction of any RT-PCR products. These findings suggest that tetracycline antibiotics selectively inhibit both mitochondrial and plastid activity.

Key words: Minocycline; Mitochondrion; Plastid; Reverse transcription-polymerase chain reaction; *Plasmodium falciparum*

1. Introduction

The increasing spread of drug-resistant *Plasmodium falciparum* is a worldwide problem for the chemotherapy of malaria. The development of new antimalarial drugs and combined use of preexisting drugs are important to overcome the drug resistance. Antimalarial activity is present in some antibiotics. Tetracycline antibiotics, such as tetracycline, doxycycline and minocycline, have been used in the treatment of drug-resistant falciparum malaria and as prophylaxis of malaria [1–3]. Tetracyclines bind specifically to the 30S ribosomes in prokaryotic cells and inhibit protein synthesis by preventing access of aminoacyl tRNA to the acceptor site of the mRNA–ribosome complex, but their activity is relatively slow against falciparum parasites [4,5]. The activity was greatly influenced by the duration of drug exposure and by oxygen tension [5]. The mode of action of tetracyclines has been suggested to act on parasite mitochondria [6]. An effect of tetracycline and doxycycline on the cytoplasmic ribosomes of the parasites has also been suggested [7].

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Abbreviations: COI, cytochrome *c* oxidase subunit I; Cyb, apocytochrome *b*; MSA1, merozoite surface antigen 1; RT-PCR, reverse transcriptation-polymerase chain reaction; rpoB/C, RNA polymerase subunits B and C; ssu rRNA, small subunit ribosomal RNA

Malaria parasites have two extrachromosomal DNAs with prokaryotic organelle-like characteristics. In *P. falciparum*, a 6 kb tandem repeated element encodes three genes identified by homology as components of the mitochondrial electron transport chain, cytochrome *c* oxidase subunits I g(COI) and III and apocytochrome *b* (Cyb) [8]. The 6 kb element also encodes small transcripts with homology to portions of organelle-like large and small subunit (ssu) rRNAs, suggesting the potential to function in ribosomes [9]. The other is a 35 kb plastid-like circular DNA, which encodes large subunit and ssu rRNAs, tRNAs, ribosomal proteins, three subunits of a eubacterial RNA polymerase (rpoA, rpoB and rpoC), a translation elongation factor and additional open reading frames [10]. The plastid genome is also present in other apicomplexan parasites, such as *Toxoplasma gondii* and *Eimeria tenella*, and the sequence data indicate that the plastid is probably of green algal origin by secondary endosymbiosis [11]. Furthermore, plastid ribosomes of *P. falciparum* were recently identified and a subset of polysomes carried plastid-specified rRNA and mRNA [12], supporting active protein synthesis in the plastid. The plastid organelle is thus focused as a new drug target in apicomplexan parasites [13,14].

In the previous study, we demonstrated that minocycline was more effective against mefloquine-, chloroquine- and pyrimethamine-resistant *P. falciparum* in vitro than tetracycline and doxycycline [15]. In the present study, we examined whether minocycline inhibits the mitochondrion and/or plastid of falciparum parasites. Since it is difficult to directly test protein biosynthesis in the malarial mitochondrion and plastid, we performed an RT-PCR assay for detection of transcriptional levels of genes encoded by the mitochondrion, plastid and nucleus.

2. Materials and methods

2.1. Cultivation of *P. falciparum* parasites

We used the SGE-1 strain, which is a drug-sensitive strain of Gambian origin, donated by Dr. P. Ambroise-Thomas of the University of Grenoble in 1979, and which has been maintained by in vitro culture alternating with occasional freezing in liquid nitrogen in our laboratory. Despite the long-term cultivation, the parasite has maintained its virulence, causing fatal sickness in *Aotus* monkeys. Cultivation of falciparum parasites was carried out according to a modified method of Trager and Jensen [16], using RPMI 1640 (Nissui Pharmaceutical, 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 [17]. Parasites were synchronized by D-sorbitol treatment and parasitized erythrocytes with a 3–4% infection rate were adjusted to a 5% packed cell volume in RPMI 1640(+) at the start of incubation. The incubator was kept

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at 37°C continuously with a gas flow mixture composed of 5% O_2, 5% CO_2, and 90% N_2.

Drugs and their sources were as follows: tetracycline hydrochloride and minocycline hydrochloride from Lederle, Japan; chloroquine sulfate from Winthrop Stearns, Manila, Philippines; pyrimethamine from Wako Pure Chemical Industries, Japan; rifampicin and thiostrepton from Sigma, USA.

2.2. Assay of RT-PCR and Southern blot hybridization

Synchronized parasites of *P. falciparum* were incubated for 14 h to enrich the young trophozoites. Infected erythrocytes, 3–4% parasite burden and 5% hematocrit, were then incubated for 4–14 h in the presence of drugs at concentrations approximately at the IC_{99} value; thiostrepton at 8 μM [18], rifampicin at 80 μM [4,18], chloroquine at 1.28 μM, pyrimethamine at 1.0 μM, tetracycline at 100 μg/ml and minocycline at 60 μg/ml. In the present study, the IC_{99} values for chloroquine and pyrimethamine were determined by growth inhibition assay for parasitemia in the presence of different concentrations of drugs for 24 h. For tetracycline and minocycline, the values were determined after incubation for 48 h. Erythrocytes in culture were collected at different time points and lysed with 0.075% saponin (Sigma) for 30 min at 4°C. After washing the cells with phosphate-buffered saline, pH 7.2, total RNA of the parasites was extracted using an RNA extraction kit (Isogene, Nippon Gene, Japan). RNA concentrations were measured at 260 nm using a spectrophotometer and RNA samples were kept at −70°C until use. One microgram of each RNA sample was treated with DNase I (Life Technologies) in 10 μl reaction mixtures. DNase-treated samples were diluted with pyrocarbonate-treated water, and appropriate amounts of RNA samples (0.1 pg–1.0 ng) underwent RT-PCR using an RT-PCR kit (SuperScript One-Step RT-PCR System, Life Technologies). The first cDNA strand was synthesized at 45°C for 30 min and denatured at 94°C for 2 min. PCR was then performed in the same tube in a 25 μl reaction mixture, containing sense and antisense primers at 10 μM, Taq DNA polymerase, 200 μM dNTP and 1.2 mM $MgSO_4$. Reaction mixtures were subjected to 15–35 cycles with 94°C for 30 s, 42°C for 30 s and 72°C for 60 s, followed by 72°C for 10 min, using PCR apparatus (Gene Amp PCR system 2400 or 9700, Applied Biosystems). The plastid DNA of *P. falciparum* contains genes encoding three subunits of a eubacterial RNA polymerase, rpoB, rpoC1 and rpoC2, and these genes are polycistronically transcribed [10]. The synthesis of the rpoB/C mRNA appears to be mediated, in part, by the plastid-encoded polymerase and the mRNA level is an indicator for assessment of the plastid activity [18]. For the RP-PCR assay, we selected two mitochondrion-encoded genes, such as the COI and Cyb genes, one plastid-encoded rpoB/C gene and two nuclear-encoded genes, such as the merozoite surface antigen (MSA1) and ssu rRNA gene [18,21,22]. The latter gene is transcribed during the asexual stage of the parasites. Primers for the PCR amplification are summarized in Table 1. Ten microliters of PCR products were electrophoresed on 2% agarose gel and transferred to a nylon membrane (Hybond-N^+, Amersham Pharmacia Biotech) in 20×SSC (1×SSC is 150 mM NaCl plus 15 mM sodium citrate, pH 7.4) for Southern blot hybridization. The PCR products were hybridized with specific oligonucleotide probes, which were labeled with digoxigenin (DIG) using a DIG oligonucleotide tailing kit (Boehringer Mannheim) (Table 1). Hybridization was performed in a hybridization solution (5×SSC, 1% nonfat milk, 0.02% SDS, 0.1% sarcosyl and 0.1 mg/ml poly(A)) at 37°C overnight, washed with a washing solution (5×SSC, 3 M tetramethylammonium chloride, 50 mM Tris–HCl, 20 mM EDTA, 0.1% SDS) at 55°C. The probes were detected immunologically using a DIG DNA detection kit, according to the manufacturer's instructions (Boehringer Mannheim).

3. Results and discussion

RT-PCR was performed to assess RNA transcription of COI and Cyb genes, encoded by the mitochondrion, rpoB/C gene encoded by the plastid, MSA1 and ssu rRNA genes encoded by the nucleus. We detected each RT-PCR product accordingly by hybridization using each specific oligonucleotide probe. The expected size of the product was 433 bp for COI, 352 for Cyb (from GenBank accession number M99416), 423 for rpoB/C (X52177), 537 for MSA1 (X52963), and 399 for ssu rRNA (M19172), respectively.

Thiostrepton binds to malarial plastid rRNA, but not to the mitochondria and cytoplasmic rRNA [24]. It impaired plastid protein synthesis, resulting in inhibition of the transcription of the rpoB/C gene [18]. Rifampicin, an inhibitor of prokaryotic RNA polymerase, also inhibited the rpoB/C gene transcription of *P. falciparum* [18]. In the present study, treatment of young trophozoites of falciparum parasites with thiostrepton and rifampicin for 8 h also specifically inhibited the transcription of the rpoB/C gene (Fig. 1A, lanes 2 and 3). For semi-quantification of the amounts of RNA transcripts, we collected RT-PCR products in different PCR cycles. Differences in the amounts of PCR products between drug-treated and non-treated materials are shown in both the linear (25 and 30 cycles) and saturated (35 cycles) range of the amplification reaction. However, treatment of parasites with thiostrepton and rifampicin caused no effect on transcription of the MSA1 and rRNA genes encoded by the nucleus, as well as COI and Cyb genes encoded by the mitochondrion (Fig. 1B–E, lanes 2 and 3). Since mitochondrial RNA polymerase genes are encoded by the nucleus [25], rifampicin appears to specifically inhibit the plastid-related transcription.

Treatment of parasites with tetracycline and minocycline for 14 h resulted in decreased hybridization signals for rpoB/C RT-PCR products in all samples collected from different PCR cycles (Fig. 1A, lanes 5 and 6). The effect of minocycline on the transcription of genes encoded by the mitochondrion was also evident. Decreased levels of COI and Cyb transcripts were detected in samples treated with mino-

Table 1
Primers and probes for RT-PCR and Southern blot hybridization

Gene	Sense and antisense primer sequence (corresponding position) [reference]	Probe sequence (corresponding position) [reference]
COI	GACCCAACATTTGCAGGAGATC (3985–4006) TTAGGTAATGCTGCCATTGATG (4396–4417) [23]	GCTATGGGATGTATAGCTG (4147–4165) [the present study]
Cyb	GCAAGTCGATATACACCAGATG (4896–4917) GGAGGAATATACTGTGAGTGATC (5226–5247) [23]	CGTTGGTTATGTCTTACCATG (5126–5146) [the present study]
rpoB/C	GGGCTTTAGAAGCTTTTGG (1841–1859) CCATTTAAAATTGGTAATCCTG (2242–2263) [18,19]	GTTTAGCTATTAATATAGAAGC (2009–2030) [18]
MSA1	GTGTGATAATATTCATGG (456–473) GGAGAGCATTTGGTG (978–992) [18,20]	AAACTTGTGTTCGGATATAG (640–659) [18]
ssu rRNA	GAACGAGATCTTAACCTGCT (1424–1443) TACTGATAAAGATTACCTA (1804–1822) [18,20]	TAACACAAGGAAGTTTAAGGC (1644–1664) [18]

COI and Cyb sequences correspond to GenBank accession number M99416, sequences or rpoB/C, MSA1 and ssu rRNA correspond to GenBank accession numbers X52177, X52963 and M19172, respectively.

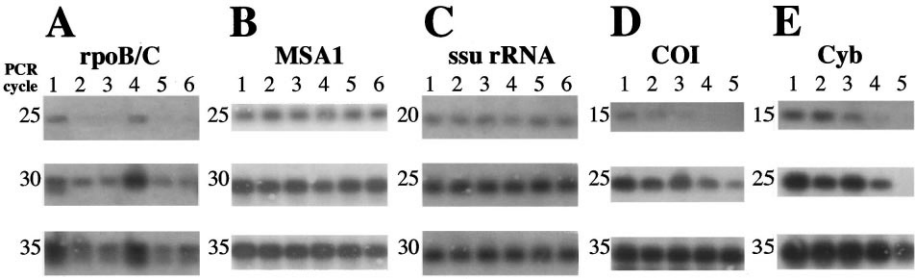

Fig. 1. Effects of minocycline and tetracycline on RNA transcription in *P. falciparum*. Total RNAs were extracted from falciparum parasites treated with the different drugs indicated below and subjected to RT-PCR assay. The RT-PCR products at different cycles (indicated on the left side of each panel) were hybridized with DIG-labeled oligonucleotide probes, corresponding to rpoB/C (A), MSA1 (B), ssu rRNA (C), COI (D) and Cyb (E) genes. A–C: lanes 1, untreated; 2, rifampicin for 8 h; 3, thiostrepton for 8 h; 4, tetracycline for 8 h; 5, tetracycline for 14 h; 6, minocycline for 14 h. D,E: lanes 1, untreated; 2, rifampicin for 8 h; 3, thiostrepton for 8 h; 4, minocycline for 8 h; 5, minocycline for 14 h.

cycline for 8 and 14 h (Fig. 1D,E, lanes 4 and 5). The effect of minocycline was dependent upon the incubation time. In contrast, despite treatment with tetracycline and minocycline for 14 h, we detected no apparent reduction of RT-PCR products in terms of MSA1 and rRNA genes, which were encoded by the nucleus (Fig. 1B,C, lanes 5 and 6). Although we did not perform a real-time PCR or competitive RT-PCR assay, we obtained reproducible inhibitory effects of minocycline. Similar results were obtained when serially diluted RNA samples were used as templates for 35 cycles of RT-PCR. In addition, we found no substantial reduction in any RT-PCR products at 25 PCR cycles in samples treated with chloroquine and pyrimethamine for 8 h (Fig. 2, lanes 1–3). These conventional antimalarial drugs do not impair directly the protein synthesis of the parasites. However, a sample treated with minocycline for 8 h showed a marked reduction of rpoB/C, COI and Cyb RT-PCR products, but the reduction was not evident for MSA1 and ssu rRNA RT-PCR products (Fig. 2, lane 4). Although a slight reduction in all transcripts was observed in the chloroquine-treated sample (Fig. 2, lane 2), we judged it a non-specific reduction from an observation of PCR products in different PCR cycles.

Tetracycline antibiotics inhibit protein synthesis in prokaryotic cells by preventing access of aminoacyl tRNA to the acceptor site of the mRNA–ribosome complex through specifically binding to the 30S ribosomes. Interestingly, a biphasic inhibition curve of protein synthesis of *P. falciparum* was noted when incorporation of [^{35}S]methionine into the tetracycline-treated parasites was monitored [7]. This phenomenon may be implied by the presence of two different types of prokaryotic translational systems, the mitochondrion and plastid in the *P. falciparum*. Decreased accumulation of rhodamine 123, an affinitive dye to the mitochondrial organelle, was reported when falciparum parasites were treated with tetracycline [6]. We also observed decreased fluorescence of rhodamine 123 in the parasites treated with tetracycline and minocycline, but not with chloroquine and pyrimethamine (unpublished data). A recent discovery of a plastid-like organelle in the apicomplexan parasites suggested that drugs and herbicides target the plastid activity [13]. Parasitocidal activity of antibacterial antibiotics may be attributed, in part, to the inhibition of plastid activity at the replication, transcription, translation or anabolic synthesis levels [14]. The malarial plastid genome encodes rRNAs, tRNAs and ribosomal proteins [10], while the mitochondrial genome encodes neither ribosomal proteins nor tRNAs, but encodes limited numbers of rRNAs [8,9]. Tetracyclines may primarily inhibit ribosome synthesis in the translational process within the plastid, eventually leading to inhibition of the synthesis of proteins, which are required for the transcription in the organelle. Since most functional proteins are suggested to be transferred to the mitochondrion from the nucleus, the growth inhibitory effect of tetracyclines may first appear in the plastid, followed by the mitochondrion.

The present study strongly suggests that tetracycline antibiotics inhibit both mitochondrial and plastid activity of *P. falciparum*. Minocycline appears to be more effective than tetracycline. Treatment with tetracycline for 8 h was inadequate for an appearance of the effect on transcription of the rpoB/C gene (Fig. 1A, lane 4), while treatment with minocycline for 8 h showed its apparent reduction (Fig. 2, lane 4). This result was coincident with findings in our previous study, in which the activity of minocycline against falciparum parasites in vitro was superior to tetracycline and doxycycline [15]. Minocycline contains two dimethylamino groups and is more lipophilic than other tetracyclines. The high inhibitory effect of minocycline on the growth of falciparum parasites may be attributable to the high lipophilicity of the drug. The lipophilic features of minocycline probably permit the high permeability through the malarial plasma membrane, mitochondrial membrane and even plastid membrane, which is composed of four bounding membranes [11,14]. Since minocycline was highly effective against not only chloroquine-resistant, but

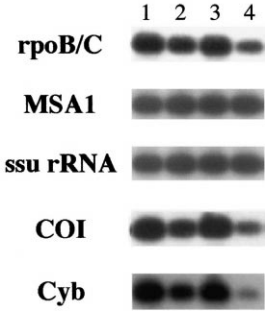

Fig. 2. Effects of chloroquine and pyrimethamine on RNA transcription in *P. falciparum*. Total RNAs were extracted from falciparum parasites treated with chloroquine, pyrimethamine and minocycline. The RT-PCR products at 25 cycles were hybridized with DIG-labeled oligonucleotide probes, corresponding to rpoB/C, MSA1, ssu rRNA, COI and Cyb genes. Lanes 1, untreated; 2, chloroquine for 8 h; 3, pyrimethamine for 8 h; 4, minocycline for 8 h.

also mefloquine- and pyrimethamine-resistant falciparum parasites [15], clinical use of this tetracycline derivative might be further considered.

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