PARTIAL PURIFICATION OF MITOCHONDRIAL DNA TOPOISOMERASE II FROM *PLASMODIUM FALCIPARUM* AND ITS SENSITIVITY TO INHIBITORS

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Abstract. Mitochondria of *Plasmodium falciparum* (K1 strain) were isolated by differential centrifugation. Mitochondrial DNA topoisomerase II from *P. falciparum* was partially purified using fast protein liquid chromatography(FPLC). Parasite mitochondria contained approximately 8% of DNA topoisomerase II activity compared with its nuclear fraction. The effects of fluoroquinolones, inhibitors of bacterial DNA topoisomerase II or DNA gyrase, against partially purified *P. falciparum* mitochondrial DNA topoisomerase II were investigated using a decatenation assay. Minimum inhibitory concentrations (MIC) of ofloxacin, ciprofloxacin and norfloxacin were >1, 10 and 100 mM, compared with that of >0.5 and 10 mM for eukaryotic DNA topoisomerase II inhibitor etoposide (VP-16) and amsacrine, respectively. The results indicate that partially purified mitochondrial DNA topoisomerase II was insensitive to fluoroquinolones and it is suggested that their inhibitory effects on *P. falciparum* growth may be directed against plastid DNA topoisomerase II.

INTRODUCTION

Widespread multidrug-resistance of *Plasmodium falciparum* calls for the urgent development of new antimalarial drugs and the investigation of potential target enzymes. DNA topoisomerases are ubiquitous and have been implicated in most biological processes that involve DNA replication, transcription and recombination (Wang, 1985). Faithful replication and segregation of chromosomes as parts of the cell division cycle involve interconversion of different topological changes in DNA which are catalyzed by DNA topoisomerases (Paul and Hickson, 1994). As these processes are essential for multiplication of all cells, inhibition of these reactions stops cellular division and growth (Liu, 1989).

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P. falciparum DNA topoisomerase II is present at all asexual stages of intraerythrocytic parasite development (Cheesman *et al*, 1994). Partially purified *P. falciparum* DNA topoisomerase II shows ATP- and Mg^{2+}-dependent activity as seen in other eukaryotes and is sensitive to eukaryotic DNA topoisomerase II inhibitors (Chavalitshewinkoon *et al*, 1994). Parasite topoisomerase II activity is also inhibited by the fluoroquinolone antibiotic ofloxacin. Fluoroquinolones have been shown to inhibit *in vitro* culture of *P. falciparum* (Divo *et al*, 1988). The specific target of fluoroquinolones is bacterial DNA topoisomerase II (DNA gyrase) which introduces negative superhelical twists into circular double-stranded DNA (Castora *et al*, 1983).

P. falciparum carries two kinds of extrachromosomal DNA: a multicopy linear 6 kb genome attributed to the mitochondrion (mtDNA) (Vaidya *et al*, 1989; Williamson *et al*, 1996) and a low copy number 35 kb circular molecule ascribed to a vestigial plastid organelle (plDNA) (Wilson *et al*, 1991). Very little is known about the enzymology of replication of the two DNA species. It is sug-

gested that the replication of *P. falciparum* mtDNA involves both a rolling circle mechanism and a recombination-associated process resembling that of bacteriophage T4 (Williamson *et al*, 1996).

Inhibition of plasmodial mitochondria topoisomerase II should lead to cessation of mtDNA replication and affect cell division. In this study, partially purified mitochondrial DNA topoisomerase II of chloroquine-resistant *P. falciparum* was tested for its sensitivity to eukaryotic and prokaryotic DNA topoisomerase II inhibitors.

MATERIALS AND METHODS

Parasite culture

Cultures of chloroquine- and pyrimethamine-resistant *P. falciparum* K1 strain were synchronized (Lambros and Vanderberg, 1979) and grown in large scale as previously described (Chavalitshewinkoon and Wilairat, 1991).

Isolation of *P. falciparum* mitochondria and nuclei

In brief, infected erythrocytes harboring mostly trophozoites and schizonts were lysed with 0.15% (v/v) saponin in phosphate-buffer saline (PBS) at 37°C for 25 minutes. Nuclei and mitochondria were then isolated from a homogenized suspension of parasites by differential centrifugation (Chavalitshewinkoon-Petmitr *et al*, 2000). The homogenate was centrifuged at 4,500*g* for 5 minutes at 4°C to remove nuclei and large membrane fragments. The supernatant fraction was carefully decanted and centrifuged at 44,700*g* for 7 minutes at 4°C, and the resulting mitochondrial pellet was resuspended in a minimal volume of homogenizing medium. Nuclear and mitochondrial fractions were stored at -80°C. Purity of mitochondria preparation was confirmed by detection of the mitochondrial marker enzyme, cytochrome *c* reductase, in each step of centrifugation (Chavalitshewinkoon-Petmitr *et al*, 2000).

Mitochondrial marker enzyme assay

Cytochrome *c* reductase activity was monitored kinetically by following the reduction of cytochrome *c* at 550 nm and 37°C using a Shimadzu 160A spectrophotometer equipped with a temperature-controlled unit. The reaction mixture, in a total volume of 1 ml, contained 200 μM cytochrome *c* from bovine heart (Sigma) in 50 mM Tris-HCl pH 8, 1 mM EDTA, 1 mM phenylmethoxysulfonylfluoride (PMSF), 0.2 mM octyl glucoside and 1 mM KCN. One unit of cytochrome *c* reductase was defined as the amount of enzyme that catalyzed the reduction of 1 μmol cytochrome *c* in 1 minute at 37°C.

Partial purification of DNA topoisomerase II from *P. falciparum* mitochondria and nuclear fractions

Parasite nuclei from $5x10^{12}$ parasites were lysed by adding 3 M KCl until the final concentration reached 0.5 M KCl and then sedimented at 100,000*g* for 40 minutes (Chavalitshewinkoon *et al*, 1993). The supernatant containing nuclear extract was dialysed against buffer A (25 mM Tris-HCl, pH 7.6, 5% (w/v) sucrose, 20% (v/v) glycerol, 1 mM PMSF, 1 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.01% NP_{40}). After dialysis, the nuclear extract was loaded onto a Resource Q (Pharmacia) anion exchange column (6 ml). DNA topoisomerase II was eluted with 0-1 M KCl linear gradient in Buffer A at a flow rate of 1 ml/min and 2 ml fractions were collected and assayed for DNA topoisomerase II activity. The active fractions were pooled and used for drug testing.

Parasite mitochondria were broken by freezing and thawing 3 times and insoluble material was removed by centrifugation at 105,000*g* for 30 minutes at 4°C. The supernatant was diluted with 10 volumes of buffer A and loaded onto a Mono Q (Pharmacia) anion exchange column (1ml). DNA topoisomerase II was eluted with 0 - 1 M KCl linear gradient in buffer A at a flow rate of 0.5 ml/min and 0.5 ml fractions were collected. The fractions containing DNA topoisomerase II activity were pooled and used for drug testing.

DNA topoisomerase II assay

DNA topoisomerase II activity was determined by a decatenation assay. Kinetoplast DNA (0.05 µg) from *Crithidia fasciculata* (Topo GEN) was used as substrate in 20 µl solution containing 40 mM Tris-HCl pH 7.9, 100 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 1 mM dithiotreitol, and 15 mg/ml bovine serum albumin (BSA). The reaction mixture was incubated at 37°C for 1 hour and reaction was stopped by the addition of 5 µl of a solution containing 5% sodium dodecylsulfate, 25% (w/v) glycerol and 0.25 mg/ml of bromophenol blue. The samples were separated on 1.2% agarose gel in Tris-borate-EDTA buffer, pH 8, containing 50 µg/100 ml of ethidium bromide at 30 volts for 13 hours. The gel was destained and photographed under UV light. One unit of DNA topoisomerase II activity was defined as the amount of enzyme that converted 0.05 µg of kDNA into 2.5 kb minicircle (opened circular and closed circular forms) in 1 hour at 37°C.

Effects of inhibitors on *P. falciparum* DNA topoisomerase II

Inhibition of DNA topoisomerase II activity was determined by preincubating the reaction mixture with various concentrations of inhibitors (amsacrine, ciprofloxacin, etoposide, norfloxacin and ofloxacin) at 37°C for 15 minutes, followed by the addition of 1 unit of enzyme. The mixture was further incubated for 1 hour at 37°C and processed according to the decatenation assay procedure described above.

Determination of protein

Protein concentration was determined by using the Bradford method (Bradford, 1976), using 1-5 µg of BSA as standards.

RESULTS

Partial purification of nuclear and mitochondrial DNA topoisomerase II from *P. falciparum*

The highest activity of cytochrome *c* reductase was found in the parasite mitochondria fraction with specific activity of 5.2 whereas those of the parasite homogenate and nuclei were 0.8 and 1.2 respectively. Partial purification of DNA topoisomerase II from nuclear and mitochondrial fractions was performed using FPLC. The detection of DNA topoisomerase II activity was based on its ability to decatenate kinetoplast DNA of *Crithidia fasciculata* into 2.5 kb minicircles, of which there were two forms: opened circular (OC) and closed circular (CC). DNA topoisomerase II activity, protein concentration and enzyme specific activity are shown in Table 1. The active fractions (5-7) of nuclear extract were eluted

Table 1
Partial purification of nuclear and mitochondrial DNA topoisomerase II from *Plasmodium falciparum* ($5x10^{12}$ parasites).

	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
Nuclei extract			
4,500*g* pellet	785	ND	ND
Resource Q eluate			
(f 5-7)	7.672	12,000	1,560
Mitochondrial extract			
44,700*g*	13.10	ND	ND
Mono Q eluate (f 7-8)	0.324	1,000	3,100

ND = not determined.

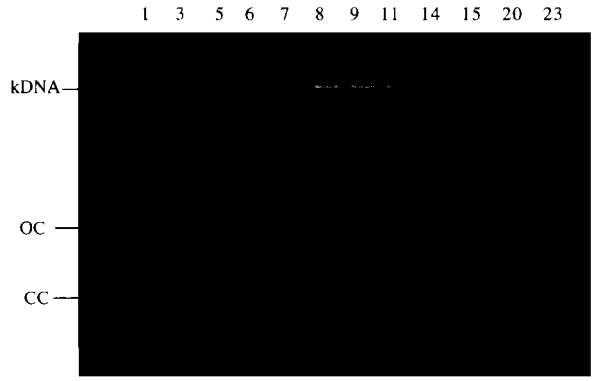

Fig 1–Decatenation activity of partially purified nuclear DNA topoisomerase II from *Plasmodium falciparum* eluted from Mono Q column with a linear gradient from 0-1 MKCl. Opened circular (OC) and closed circular (CC) molecules are indicated.

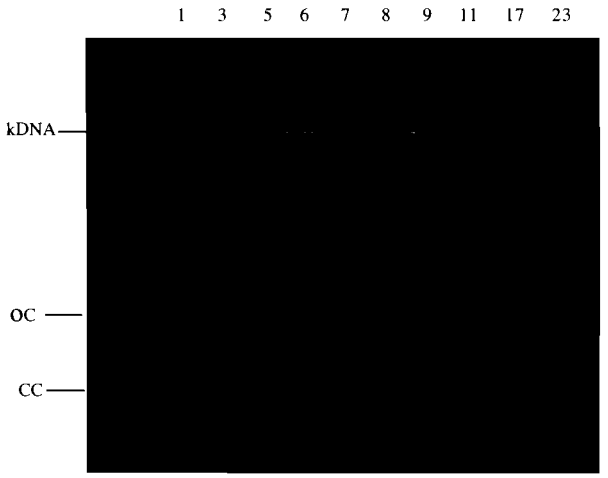

Fig 2–Decatenation activity of partially purified mitochondrial DNA topoisomerase II from *Plasmodium falciparum* eluted from Mono Q column with a linear gradient from 0-1 MKCl. Opened circular (OC) and closed circular (CC) molecules are indicated.

from anion exchange column by KCl in the range of 200-300 mM (Fig 1). The pooled active fractions contained 12,000 units of DNA topoisomerase activity with a specific activity of approximately 1,500 units/mg protein. A Mono Q anion-exchange column was used to obtain partially purified *P. falciparum* topoisomerase II from mitochondrial extract. The active fractions (7 and 8) were eluted with 150-250 mM KCl (Fig 2) and contained 0.32 mg of protein with 1,000 units of DNA topoisomerase II activity, approximately 8% of the total activity present in nuclear extract. The specific activity of *P. falciparum* mitochondrial topoisomerase II was about twice that obtained from the nuclear extract. Mitochondrial DNA topoisomerase II was not stable, with 40% loss in activity after 2 months at -80°C even in the presence of protease inhibitors during the isolation and purification steps.

Effects of prokaryotic and eukaryotic DNA topoisomerase II inhibitors on mitochondrial DNA topoisomerase II from *P. falciparum*

Inhibition of decatenation activity of partially purified mitochondria DNA topoisomerase II from *P. falciparum* was investigated using both prokaryotic and eukaryotic DNA topoisomerase II inhibitors (Table 2). The minimum inhibitory concentration (MIC) of amsacrine, a topoisomerase II inhibitor, was equal to that of the gyrase inhibitor ciprofloxacin and was one tenth of that of norfloxacin. Complete inhibition of decatenation was not achieved with 0.5 mM etoposide nor with 1 mM ofloxacin; limited solubility hampered the investigation of higher concentrations.

DISCUSSION

Although only one mitochondrion organelle is found in asexual form of *P. falciparum* (Slomianny and Prensier 1986), *in vitro* large scale cultivation of *P. falciparum* made this study possible by allowing a high quantity of malarial parasites ($5x10^{12}$) to be obtained. Mitochondria of *P. falciparum* were successfully isolated and detection of cytochrome *c* reductase at each step of isolation confirmed the purity of mitochondria organelles. A previous study showed that 7-fold purification and a yield of 10 % of mitochondria could be obtained by differential centrifugation (Chavalitshewinkoon-Petmitr *et al*, 2000). Mitochondrial DNA topoisomerase II was partially purified using

Table 2
Minimum inhibitory concentration of DNA topoisomerase II inhibitors against decatenation activity of one unit of *P. falciparum* mitochondrial DNA topoisomerase II compared with partially purified cellular extract enzyme.

Inhibitors	Minimum inhibitory concentration (mM)	
	Mitochondrial DNA topoisomerase II	cellular extract DNA topoisomerase II[a]
Amsacrine	10	1
Ciprofloxacin	10	ND
Etoposide (VP-16)	0.5[b]	0.13
Norfloxacin	100	ND
Ofloxacin	>1 [b]	0.01

[a]Taken from Chavalitshewinkoon *et al* (1994).
[b]Complete inhibition was not detected at this concentration.
ND = not determined.

FPLC. The amount of mitochondrial DNA topoisomerase II was approximately 8% of the enzyme obtained from the nuclear extract, in keeping with the other studies showing DNA topoisomerase II constitutes a major protein of the nuclear matrix (Fernandes and Catapano, 1995).

P. falciparum mitochondrial DNA topoisomerase II exhibited surprisingly comparable sensitivity to both eukaryotic DNA topoisomerase II and DNA gyrase inhibitors. Moreover, the parasite mitochondrial enzyme was less sensitive to these inhibitors than its nuclear counterpart (Chavalitshewinkoon *et al*, 1994): the MIC of ofloxacin against *P. falciparum* topoisomerase II from parasite total extract was 0.01 mM compared with >1 mM for the mitochondrial enzyme (Table 2).

Our results prompt the making of two points: firstly, a selective and high accumulation of fluoroquinolones in *P. falciparum* mitochondria is needed to inhibit mtDNA toposomerase II; secondly, as the enzyme was not susceptible to these inhibitors, mtDNA topoisomerase II may not be a good target for fluoroquinolones. The possible target may be the DNA topoisomerase II of the plastid. Weissig *et al* (1997) have reported that the eukaryotic topoisomerase II inhibitor VP-16 induces cleavage of both nuclear and 35-kb DNA of *P. falciparum* whereas ciprofloxacin, a fluoroquinolone drug known to act on the bacterial type II topoisomerase II or DNA gyrase, only induces cleavage of the 35-kb DNA.

Our results and those of Weissig *et al* (1997) suggest that *P. falciparum* contains two or more pharmacologically distinguishable topoisomerase II activities. Whether these pharmacologically distinct activities represent isoforms of topoisomerase II is unclear. So far, only a single topoisomerase II gene has been identified in *P. falciparum* and evidence suggests that it encodes a nuclear topoisomerase II protein with an apparent mass of 160 kDa (Cheesman *et al*, 1994). However, by using monoclonal antisera against extracted DNA topoisomerase II of *Crithidia fasciculata*, kinetoplast protein of both *Trypanosoma cruzi* and *Crithidia fasciculata* are recognized whereas monoclonal antisera against recombinant DNA topoisomerase II of *Crithidia fasciculata* shows that enzyme is located exclusively in the nucleus of the parasite (Fragoso *et al*, 1998). In order to prove that *P. falciparum* contains two or more distinguishable DNA topoisomerase II, the monoclonal antisera against both PfTOP2 gene and corresponding enzyme extracted from the parasite must be performed and localization of the enzyme will be detected by confocal laser microscopy.

However, the present study raises the interesting possibility that fluoroquinolone gyrase inhibitors might be mediating their antimalarial effects by interfering with DNA topoisomerase II activity that is preferentially associated with plastids rather than mitochondria. To prove this, plastid DNA topoisomerase II from *P. falciparum* needs to be isolated. Unfortunately, isolation of plastids from malaria parasites has not been accomplished. Nevertheless, plastid DNA topoisomerase II could provide a potential novel and unique target for the development of future antimalarial drugs.

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