PARTIAL PURIFICATION AND CHARACTERIZATION OF *TRICHOMONAS VAGINALIS* DNA TOPOISOMERASE II

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Abstract. DNA topoisomerases regulate conformational changes in DNA topology by catalyzing the breakage and rejoining of DNA strands during the cell cycle. These processes are essential for the multiplication of cells, and inhibition of these reactions stops cell division and cell growth. Drug resistance to *Trichomonas vaginalis*, a common sexually transmitted protozoan parasite, is increasing worldwide, and DNA topoisomerase II may provide a new target for anti-trichomonal drug development. In this study, *T. vaginalis* DNA topoisomerase II was partially purified from a large scale axenic culture using fast protein liquid chromatography with a yield of 0.16% and 17-fold purification. The partially purified enzyme was strictly dependent on ATP and Mg^{2+} with optimal concentration of 1 and 10 mM respectively for relaxation activity. *T. vaginalis* DNA topoisomerase II activity was inhibited by m-amsacrine (m-AMSA) and ofloxacin at minimum inhibitory concentration (MIC) of 250 μM. At this concentration, ciprofloxacin showed incomplete inhibition whereas metronidazole was inactive. DW6, a DNA quadruplex binder, was the most active compound with MIC of 62.5 μM, suggesting the potential for development of such compounds as selective anti-trichomonal drugs in the future.

INTRODUCTION

Trichomonas vaginalis is a protozoon causing common sexually transmitted infection, which is increasingly recognized as an important infection in women and men worldwide. Recent estimates have suggested that *T. vaginalis* infections account for over 170 million cases per year (Shafir *et al*, 2009). *T. vaginalis* is recognized as a common cause of vaginitis and as a factor contributing to preterm birth and low birth weight (Cotch *et al*, 1997; Klebanoff *et al*, 2001). *T. vaginalis* infections have also been linked with increased transmission of human immunodeficiency virus (HIV) (Sorvillo *et al*, 2001; Kissinger *et al*, 2008). Trichomoniasis may increase the risk of HIV-1 acquisition by increasing susceptibility to bacterial vaginosis or persistence of abnormal vaginal flora (Moodley *et al*, 2002a). Also, *T. vaginalis* can cause pelvic inflammatory disease in both HIV-infected and -uninfected women (Moodley *et al*, 2002b).

Metronidazole has been used as the drug of choice for *T. vaginalis* infection since 1960, but recently, clinical resistance to metronidazole has been reported (Petrin *et al*, 1998; Schmid *et al*, 2001; Upcroft *et al*, 2006). Most of the treatment failures are caused by *T. vaginalis* strains with lowered susceptibility to metronidazole. Typically, resistant iso-

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lates show low susceptibility to the drug *in vivo* and under anaerobic conditions *in vitro*. The prevalence of metronidazole and tinidazole resistant *T. vaginalis* seems to be increasing (Sobel *et al*, 1999; Schwebke and Barrientes 2006). Furthermore, concern about mutagenic and carcinogenic potential of metronidazole has been raised (Johnson *et al*, 1993).

In the cell, DNA topology is regulated and controlled by DNA topoisomerases, which break and reseal the phosphodiester bonds of DNA allowing the passage of the strands of DNA through the transient gaps. Based on their mechanism of action, there are two types of DNA topoisomerases. DNA topoisomerase I alters the pitch of DNA double helix by cutting one DNA strand and allowing passage of the complementary strand through the transient nick. DNA topoisomerase II requires ATP hydrolysis for catalytic activity and changes DNA topology by creating transient double strand breaks through which a second intact double helix is passed. Topoisomerase function is required for various DNA transactions, such as replication, transcription, recombination and repair of DNA and also for chromosome condensation and sister chromatid segregation (Wang *et al*, 1996; Nitiss *et al*, 1998). In the search for more effective chemotherapy treatments, protozoan topoisomerases are now being considered as potential drug targets, building on the clinical success of anticancer and antibacterial agents that target human and bacterial topoisomerases (Giles and Sharma, 2005; Tse-Dinh, 2009).

In this study, DNA topoisomerase II from *T. vaginalis* extract was partially purified by FPLC, characterized and tested with a number of known DNA topoisomerase II inhibitors and a DNA quadruplex binder. Results obtained from this study may lead to future design and synthesis of potential antitrichomonal agents.

MATERIALS AND METHODS

Parasite culture

T. vaginalis used in this study was a local strain previously cultured and cloned (Arthan *et al*, 2008) using agar plate culture technique (Hollander, 1976). The axenic culture was maintained continuously using TYM medium (Diamond, 1957). A large scale culture of *T. vaginalis* was started by transferring 0.7 ml of *T. vaginalis* containing approximately $1x10^5$-$1x10^6$ trophozoites/ml to 70 ml of TYM medium in 125 ml screw capped Erlenmeyer glass flask and supplemented with 7 ml of human plasma, 0.25 μg/ml chloramphenicol and 50 μg/ml gentamicin sulfate. The culture was incubated at 37ºC in a humidified atmosphere of 5% CO_2. *T. vaginalis* in the logarithmic phase of growth was harvested within 24 hours of initiation of culture. Cell suspension was washed three times with phosphate buffered saline (PBS), pH 7.6 and centrifuged at 8,000*g* for 10 minutes at 4ºC. Cell pellet was kept at -80ºC until used.

Partial purification of *T. vaginalis* DNA topoisomerase II

All purification steps were conducted on ice or at 4ºC. Approximately 31.4 ml of packed parasites were suspended in 17.5 ml of extraction buffer (50 mM Tris HCl pH 7.6, 1 mM EDTA, 2 mM dithiotritol (DTT), 0.01% Nonidet P_{40} (NP_{40}), 1 mM phenyl methyl sulfonyl fluoride (PMSF)), broken in a Dounce homogenizer and diluted with an equal volume of dilution buffer (50 mM Tris HCl pH 7.6, 1 mM EDTA, 2 mM DTT, 20% (w/v) sucrose, 0.01% NP_{40}, 1 mM PMSF). To extract nucleoproteins, 3 M KCl were added with stirring until a final concentration of 0.5 M KCl was obtained. After further stirring for 30 minutes, the suspension was centrifuged at 100,000*g* for 40 minutes at 4ºC. The supernatant was dialyzed overnight against buffer A (25 mM Tris HCl pH 8.0, 1 mM DTT,

5% sucrose, 20% glycerol, 0.01% NP_{40}, 1 mM EDTA, 1 mM PMSF) and designated Fraction I.

Purification steps were performed at 4ºC using an automated FPLC system (Pharmacia). Fraction I was loaded onto a 6 ml Resource Q column (Pharmacia) equilibrated with buffer A. The column was washed with 216 ml of buffer A and proteins were eluted with a 72 ml of linear gradient (0-1 M KCl in buffer A) at a flow rate of 2 ml/minute, and 2 ml fractions were collected. The salt concentration across the gradient and absorbance at 280 nm were recorded. Fractions were assayed as described below. The active fractions, 7-21, eluting between 0.09-0.51 M KCl were pooled and designated as fraction II. Fraction II was dialyzed overnight against buffer B (buffer A adjusted to pH 7.6) and loaded onto a 6 ml Resource S column. The column was washed with 48 ml of buffer B and protein was eluted with a 48 ml of linear gradient (0-1 M KCl in buffer B) at a flow rate of 1.5 ml/minute and 1.5 ml fractions were collected. The active fractions, 9-18, eluting between 0.14-0.48 M KCl were pooled and designated as fraction III. Fraction III was dialyzed against buffer C (5 mM potassium phosphate buffer pH 7.6, 100 mM KCl, 1 mM EDTA, 5% sucrose, 20% glycerol, 1 mM PMSF, 1 mM DTT and 0.01% NP_{40}) and applied to a 5 ml Hitrap Heparin column (Pharmacia). The column was washed with 6 ml of buffer C and protein was eluted with 8 ml of linear gradient (0.005 M - 0.60 M potassium phosphate buffer pH 7.6 in buffer C) at a flow rate of 0.5 ml/minute and 0.5 ml fractions were collected. The active fractions, 5-14, were eluted at 0.05 - 0.52 M potassium phosphate buffer pH 7.6 in buffer C and designated as fraction IV (5 ml). Fraction IV was dialyzed against buffer D 25 mM Tris HCl (pH 7.6), 0.1 M KCl, 1 mM EDTA, 1 mM PMSF, 1 mM DTT 5%, sucrose, 20% glycerol and 0.01% NP_{40}, and applied to 1 ml single-stranded DNA (ssDNA) column and eluted with a 9 ml of linear 0.1-1 M KCl gradient in buffer D at a flow rate of 0.25 ml/minute and 0.25 ml fractions were collected. Active fractions V (0.33-0.67 M KCl) were designated as fraction V (2.75 ml). They were diluted 2 fold with buffer B and then loaded onto 1 ml Hitrap Blue column (Pharmacia), pre-equilibrated with buffer B. *T. vaginalis* DNA topoisomerase II was eluted with a 7 ml of linear 0-1 M KCl gradient in buffer B at a flow rate of 0.2 ml/minute and 0.4 ml fractions were collected. Active fractions (0.45-0.66 M KCl) were designated as fraction VI (3.2 ml). Fraction VI was diluted ten fold with buffer B and loaded onto 1 ml Mono Q column (Pharmacia), equilibrated with buffer B and eluted with a 10 ml of linear 0.1-0.8 M KCl gradient in buffer B at a flow rate of 0.25 ml/minute and 0.3 ml fractions were collected. Fractions 18-27 eluting at 0.33-0.64 M KCl showed highest type II DNA topoisomerase activity and were pooled and designated as fraction VII (3 ml). The enzyme could be stored at -80ºC for three months without loss of activity. Amount of protein in each fraction was measured using Bradford method (Bradford, 1976).

DNA topoisomerase II assay

Relaxation activity of DNA topoisomerase II was determined by detecting the conversion of supercoiled plasmid DNA to its relaxed form at the presence of ATP (Miller *et al*, 1981). DNA topoisomerase II assay was performed in 18 µl of reaction mixture containing 50 mM Tris-HCl buffer pH 8.0, 0.12 M KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, 0.25 µg of supercoiled pUC19 DNA (Fermentas, Canada) and 2 µl of each fraction. The mixture was incubated at 37ºC for 30 minutes and reaction was terminated by adding 5 µl of stop solution (5% SDS, 25% w/v glycerol and 0.25 mg/ml of bromophenol blue). The sample was subjected

to 1% agarose gel-electrophoresis in 1xTAE (Tris-acetate-EDTA buffer) pH 8.0 at 30 volts for 6 hours. The agarose gel was stained with 0.5 μg/ml ethidium bromide and DNA product was visualized on a UV transilluminator.

One unit of DNA topoisomerase II activity is defined as the amount of enzyme capable of relaxing 0.25 μg of supercoiled DNA in 30 minutes at 37ºC.

Requirements of *T. vaginalis* DNA topoisomerase II activity

The requirements of DNA topoisomerase activity were determined by adding ATP at concentrations ranging from 0.01-128 mM, $MgCl_2$ at concentrations ranging from 0.02-80 mM, and KCl of concentrations ranging from 15-420 mM.

Effects of DNA topoisomerase inhibitors on *T. vaginalis* DNA topoisomerase II

Known DNA topoisomerase II inhibitors, such as m-amsacrine (m-AMSA), ofloxacin and ciprofloxacin (Sigma-Aldrich, USA), were tested comparing to metronidazole and a quadruplex binder, DW6 (Fig 1), kindly provided by Prof William A Denny, Auckland Cancer Society Research Centre, Faculty of Medical and Health Sciences, The University of Auckland, New Zealand. Compounds were diluted with 10 mM Tris-HCl pH 8. Inhibitory effects were determined by adding 2 μl of inhibitors at various concentrations to 15 μl of reaction mixtures. pUC19 plasmid DNA was incubated with various concentrations of test compounds for 10 minutes prior to conducting DNA topoisomerase II assay. A 3 μl aliquot of *T. vaginalis* DNA topoisomerase II was added and the reaction mixture was incubated at 37ºC for 30 minutes and then assayed for DNA topoisomerase II activity. The highest concentration of DMSO was used as negative control. Initial experiments were performed with 10-fold dilutions of stock concentration. After obtaining minimum inhibitory concentration(MIC) experiments were performed by expanding the drug concentrations around MIC.

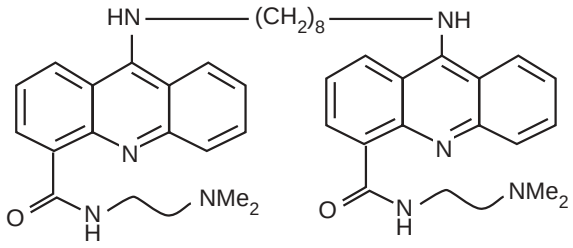

Fig 1–Chemical structure of DNA quadruplex binder, DW6.

RESULTS

Partial purification and characterization of *T. vaginalis* DNA topoisomerase II

The substrate used to measure *T. vaginalis* DNA topoisomerase II activity during the purification step and characterization of DNA topoisomerase II was a supercoiled plasmid DNA (pUC19 DNA) in the presence of ATP. DNA topoisomerase II activity of *T. vaginalis*, protein concentration, specific activity and yield after each purification step are shown in Table 1. *T. vaginalis* DNA topoisomerase II was partially purified to about 17-fold purification with a final yield of 0.16% and a specific activity of $8.8x10^3$ units/mg from 31.4 ml of packed parasites. Nuclease activity was clearly observed during the purification steps using several columns (Fig 2) and this is one factor that affected the purification of *T. vaginalis* DNA topoisomerase II to near homogeneity.

The relaxation activity of *T. vaginalis* DNA topoisomerase II was detected in the presence of at least 0.25 mM ATP in the assay and the highest activity of the enzyme required 1.0 mM ATP (Fig 3). The effective

Table 1
Partial purification of *Trichomonas vaginalis* DNA topoisomerase II from crude extract using FPLC.

Purification step	Total protein (mg)	Total activity (unit)	Specific activity (units/mg)
Crude extract	1,420.42	ND	ND
Resource Q (f 7-21)	37.36	2.0×10^4	5.3×10^2
Resource S (f 9-18)	2.91	7.5×10^3	2.6×10^3
HiTrap Heparin (f 5-14)	1.16	4.0×10^3	3.4×10^3
ss-DNA (f 14-24)	0.37	1.4×10^3	3.8×10^3
HiTrap Blue (f 26-33)	0.13	0.6×10^3	4.6×10^3
Mono Q (f 18-27)	0.06	0.5×10^3	8.8×10^3

f, fraction number; ND, not determined

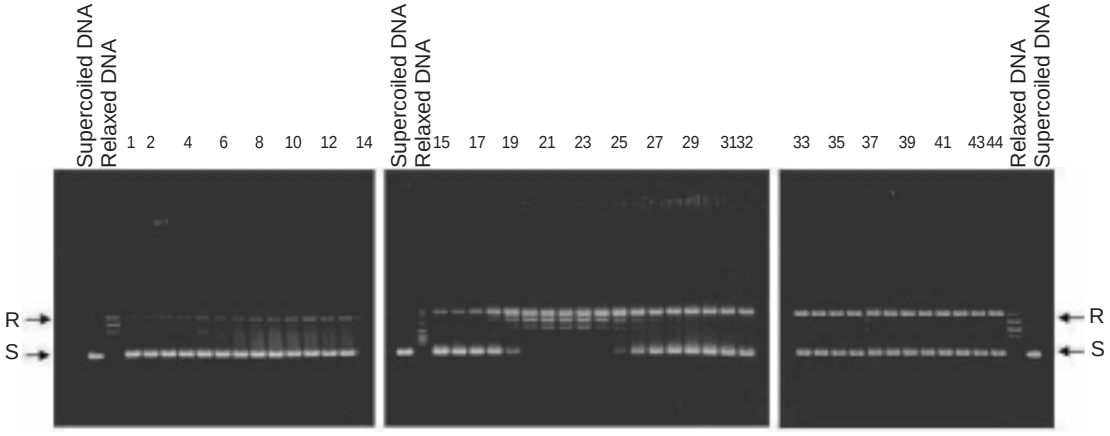

Fig 2–Relaxation activity of partially purified *T. vaginalis* DNA topoisomerase II eluted from Mono Q column. DNA topoisomerase II assay was performed in 18 µl of reaction mixture containing 50 mM Tris-HCl buffer pH 8.0, 0.12 M KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, 0.25 µg of supercoiled pUC19 DNA and 2 µl of each fraction. The mixture was incubated at 37ºC for 30 minutes and reaction was terminated by adding 5 µl of stop solution (5% SDS, 25% w/v glycerol and 0.25 mg/ml of bromophenol blue). Active fraction numbers 18-27 show supercoiled DNA relaxing activity of DNA topoisomerase II. (R, relaxed form; S, supercoiled form)

concentration of KCl in the assay was in the range of 60 - 210 mM KCl and optimum concentration of KCl was 120 mM (Fig 4). The effect of divalent cations on the relaxation activity was determined upon addition of various concentrations of $MgCl_2$. The minimum Mg^{2+}-requirement was 5 mM and the highest enzyme activity was observed at 10 mM $MgCl_2$ (Fig 5). The optimal pH was about 8 (data not shown).

Effects of DNA topoisomerase inhibitors on *T. vaginalis* DNA topoisomerases II

Pre-incubation of pUC19 DNA with various concentrations of drugs for 10 minutes caused a concentration-dependent inhibition

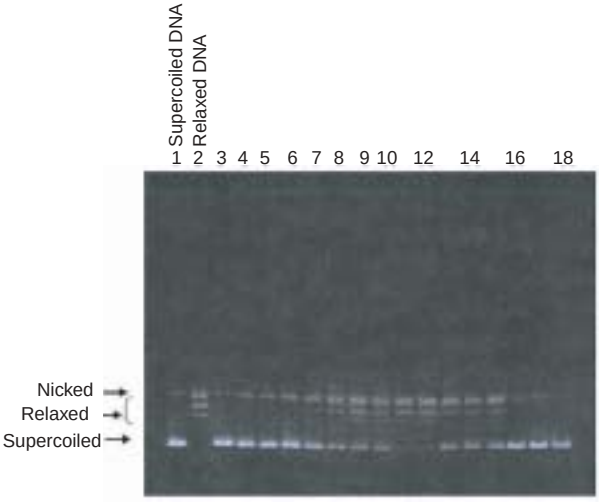

Fig 3–Effect of ATP on DNA relaxation activity of partially purified *T. vaginalis* DNA topoisomerase II. DNA relaxation assay was conducted as described in legend of Fig 1. Lane 1, negative control (no enzyme); lane 2, positive control (with enzyme); lanes 3 -18 with 0, 0.01, 0.02, 0.03, 0.06, 0.13, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64 and 128 mM ATP, respectively.

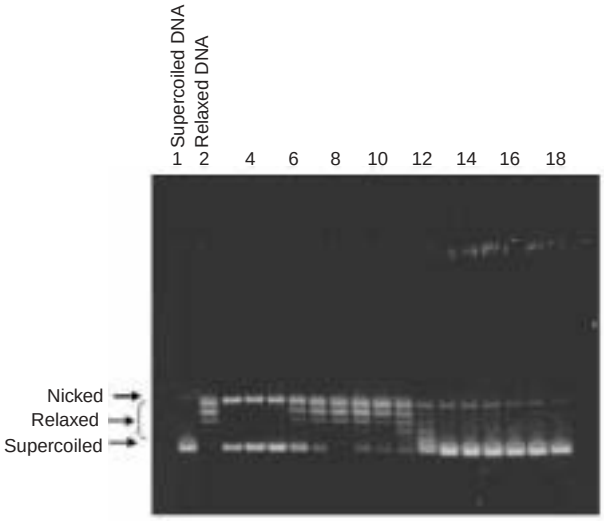

Fig 4–Effect of ionic strength on DNA relaxation activity of partially purified *T. vaginalis* DNA topoisomerase II. DNA relaxation assay was conducted as described in legend of Fig 1. Lane 1, negative control (no enzyme); lane 2, positive control (with enzyme), lanes 3 -18 with 0, 15, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390 and 420 mM KCl, respectively.

Table 2
Effects of topoisomerase II inhibitors and a synthetic DNA intercalator, DW 6, on relaxation activity of partially purified *T. vaginalis* DNA topoisomerase II.

Compound	Relaxation inhibiton MIC (μM) [a]
Metronidazole	N.I.[b]
DNA topoisomerase II inhibitor	
m-AMSA	250
Ciprofloxacin	N.I.[c]
Ofloxacin	250
DW6	62.5

[a]MIC value was determined from the lowest drug concentration which inhibits relaxation activity of the enzyme.
[b]N.I., no inhibition at 250 μM.
[c]N.I., no complete inhibition at 250 μM.

of *T. vaginalis* DNA topoisomerase II activity (Table 2). The MIC value of m-AMSA, ofloxacin and DW6 was 250, 250 and 62.5 μM, respectively. DW6 showed the lowest MIC (62.5 μM) against *T. vaginalis* DNA topoisomerase II activity.

DISCUSSION

In this study, *T. vaginalis* DNA topoisomerase II was successfully (partially) purified using seven columns. The low fold purification and yield obtained were most likely due to the presence of trichomonal proteases, despite the addition of PMSF, glycerol and sucrose in all steps of enzyme purification. The major difficulty in measuring topoisomerase II activity in crude extract of eukaryotic cells is the abundance of nucleases that could destroy substrate. How-

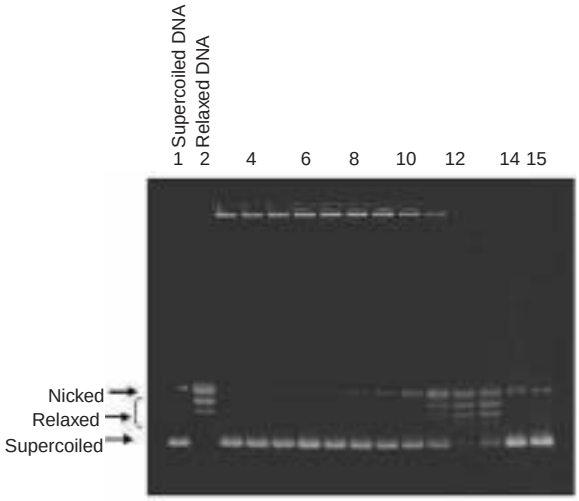

Fig 5–Effect of magnesium concentration on DNA relaxation activity of partially purified *T. vaginalis* DNA topoisomerase II. DNA relaxation assay was conducted as described in legend of Fig 1. Lane 1, negative control (no enzyme); lane 2, positive control (with enzyme); lanes 3 -15, with 0.02, 0.04, 0.08, 0.16, 0.31, 0.63, 1.25, 2.5, 5, 10, 20, 40 and 80 mM $MgCl_2$, respectively.

ever, the partially purified *T. vaginalis* enzyme was still active when stored at -80ºC for three months without loss of activity.

Enzyme activity was detected using a DNA relaxation assay in which topoisomerase II catalyzes an ATP-dependent DNA relaxation. This relaxation assay has been proven to be useful in purification procedures separating type I and type II topoisomerase from eukaryotic sources. Eukaryotic type I and II topoisomerases often copurify in chromatographic purification schemes and the relaxation assay has been used to distinguish ATP-independent topoisomerase I activity from ATP-dependent topoisomerase II activity (Miller *et al*, 1981).

DNA topoisomerase II of *T. vaginalis* showed ATP and Mg^{2+} -dependent activity as in other eukaryotes (Wang, 1985). A 172 kDa topoisomerase II is found in HeLa cells (Miller *et al*, 1981), whereas topoisomerase II from yeast is purified as a "polydisperse" band of 150 kDa, indicating that some proteolysis had occurred (Shelton *et al*, 1983). In calf thymus, bands of 125 and 140 kDa are found, but these clearly represent proteolytic products of a 180 kDa form detected by immunoblotting of cell lysate (Halligan *et al*, 1985). Tryptic analysis of the two proteins showed no significant differences. A more recent purification of the enzyme from calf thymus found protein bands of 175 and 150 kDa (Schomburg and Grosse, 1986) and while no comparison of the two bands was made, the authors did state that antibodies raised against the 175 kDa protein cross reacted with the 150 kDa protein. In protozoa, 160 kDa topoisomerase II was isolated from *Plasmodium berghei* (Riou *et al*, 1986). From analysis of *T. vaginalis* genome, a putative *T. vaginalis* DNA topoisomerase II is encoded by an open reading frame of 4,466 base pairs, which predicts a protein of 1,458 amino acids with an anticipated molecular weight of 167 kDa (Miller *et al*, 1981).

Unwinding of the double strands of the DNA helix is a hallmark feature of intercalating drugs, such as chloroquine, m-AMSA and ethidium bromide (Wang, 1985). The degrees of supercoiling produce distinct forms of circular DNA known as topoisomers. Topoisomers can be separated as discrete bands using gel electrophoresis as resistance to migration through such gels decreases with increases in degree of supercoiling. As ATP-dependent DNA topoisomerase causes stepwise relaxation of supercoiled DNA substrate, this action results in a progressive decrease in the number of superhelical turns, thus generating a population of partially relaxed forms. However, in order to produce a system with maximal sensitivity to the inhibitory effects of compounds, assay conditions were adopted such that in most cases

all supercoiled pUC19 DNA molecules were converted to the fully relaxed form in 30 minutes at 37ºC, even though some nicked circular DNA was present as a contaminant in the pUC19 preparation used.

At a concentration of 250 μM, metronidazole was unable to inhibit the DNA relaxation activity of *T. vaginalis* topoisomerase II whereas ciprofloxacin showed incomplete inhibition. The most potent inhibitor was the quadruplex binder, DW6, which could inhibit *T. vaginalis* growth with MIC of 12.5 μM (data not shown). Ofloxacin has been reported with MIC as high as 960 μM against the same parasite strain (Chavalitshewinkoon-Petmitr *et al*, 2000). Interestingly, it has been demonstrated that human DNA topoisomerase IIβ binds preferentially to a four-way junction DNA (West and Austin, 1999), supporting the notion that *in vivo* DNA topoisomerase II interacts preferentially with DNA crossovers. Further exploration of quadruplex binders and bisintercalators, so as to enhance their capacity for stabilising DNA crossovers, may provide new approaches to the development of novel poisons of topoisomerases for use as antiprotozoal agents.

As *T. vaginalis* DNA topoisomerase II could not be purified to homogeneity, cloning and expression of *T. vaginalis* will be the approach in the future in order to study its biochemical and physiological properties. Nevertheless, a partial purified *T. vaginalis* DNA topoisomerase II from this study provides materials for testing of potential drugs as new antitrichomonals.

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