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Short communication

Evidence for the presence of R250G mutation at the ATPase domain of topoisomerase II in an arsenite-resistant *Leishmania donovani* exhibiting a differential drug inhibition profile

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ABSTRACT

Resistance to operational drugs is a major barrier to successful antileishmanial chemotherapy that demands development of novel drug intervention strategies based on rational approaches. Model drug resistance phenotypes, such as arsenite resistance used in the current study, facilitate our understanding of the mechanism of drug resistance and assist in identifying new drug target(s). The current study was undertaken to investigate the sensitivity of topoisomerase II (topo II) of arsenite-sensitive (Ld-Wt) and -resistant (Ld-As20) *Leishmania donovani* to antileishmanial/anti-topo II agents. The effect of antileishmanial/anti-topo II drugs on partially purified topo II enzyme from Ld-Wt and Ld-As20 revealed differential inhibition of topo II decatenation activity for the two strains, with a lower amount of drug required to inhibit activity by 50% in Ld-Wt compared with Ld-As20. Comparison of topo II sequences from both strains indicated a point mutation, R250G, in the ATPase domain of the resistant strain. Furthermore, the Arg-250 of the ATPase domain of topo II was observed to be conserved throughout different species of *Leishmania*. Variation in the topo II gene sequence between Ld-Wt and Ld-As20 is envisaged to be responsible for the differential behaviour of the enzymes from the two sources.

1. Introduction

In kinetoplastids such as *Leishmania*, complex kinetoplast DNA (kDNA) poses a unique topological problem that requires topoisomerase II (topo II) activity and presents an interesting cellular target [1]. This led to topo II being studied and cloned in an array of parasites, including *Leishmania* [1]. Our previous investigations revealed overexpression and increased activity of topo II in an arsenite-resistant *Leishmania donovani* strain displaying a multidrug-resistant (MDR)-like phenotype [2]. Recently we have reported this enzyme as ATP-dependent topo II since it displayed a ladder of topoisomers in a relaxation assay. The decrease in decatenation activity at lower ATP concentration (1 mM) and its inhibition by novobiocin further confirmed it as an ATP-dependent topo II enzyme [3]. Furthermore, the enzyme activity in nuclear extract was found to be resistant to different topo II inhibitors such as novobiocin [2], pentamidine and doxorubicin [3] and actinomycin D (unpublished data). In the current study, we have attempted to monitor the effect of these drugs on partially purified topo II enzymes from arsenite-sensitive (Ld-Wt) (strain MHOM/80/IN/Dd8) and arsenite-resistant (Ld-As20) strains of *L. donovani*.

2. Materials and methods

2.1. Topo II decatenation assay

Topo II was assayed as described previously [2,3]. Relative densitometric analysis was performed using a gel documentation system (Gel Doc 2000; Bio-Rad, Hercules, CA) with Quantity One® software (Bio-Rad). The density of the free minicircle DNA band released at 4 μg of Ld-As20 topo II was arbitrarily given a value 1.0 and relative densities of the other bands were derived.

2.2. Polymerase chain reaction (PCR) amplification and sequencing of the topo II gene

Leishmania donovani genomic DNA was isolated as described by Singh and Dey [2]. PCR was carried out using an Expand Long Template PCR System (Roche Applied Science, Mannheim, Germany)

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Table 1
Polymerase chain reaction (PCR) primers used in *LdTOP2* amplification and sequencing

		Start position in *LdTOP2* gene (bp)
Amplification primers		
Primer 1 (forward)	5′-GGGAATTCCATATGACAGACGCTTCCAAG-3′	1
Primer 2 (reverse)	5′-CTCGAGCATCAAAACATGTGGCAGCAGACGA-3′	3708
Sequencing primers		
Primer 3 (forward)	5′-GAACAAGGAGATCGGCGCGGG-3′	1194
Primer 4 (reverse)	5′-CTGTAATGAGCGTGCGGATGTTC-3′	1318
Primer 5 (forward)	5′-CGAGGAGGGCACTTTCGTGGAGC-3′	2400
Primer 6 (reverse)	5′-GAGCGGATGGAATGACGGGATCG-3′	2520
M13 forward	5′-GTAAAACGACGGCCAGT-3′	
M13 reverse	5′-CAGGAAACAGCTATGAC-3′	

using 500 ng of DNA as template in a final reaction volume of 50 μL containing 500 μM of each dNTP, 300 nM of each forward and reverse gene-specific primers designed as per the reported topo II sequence of *L. donovani* [1] (GenBank accession no. AF150876) (Table 1), 1 × PCR buffer and 3.75 U of enzyme. PCR amplification was carried out for 30 cycles (10 cycles at 94 °C for 10 s, 65 °C for 30 s and 68 °C for 4 min, and 20 cycles at 94 °C for 10 s, 60 °C for 30 s and 68 °C for 4 min, with 20 s extension in each cycle) in a DNA thermal cycler (Bio-Rad). For topo II sequencing, PCR was carried out for 24 cycles (96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min) using Ld-Wt and Ld-As20 topo II constructs (see Table 1 for primers used for sequencing). The PCR-amplified DNA was precipitated with ethanol and ethylene diamine tetra-acetic acid (EDTA). The pellet was washed with 70% ethanol and air-dried. DNA was denatured following the addition of Hi-Di™ formamide (Applied Biosystems, Foster City, CA). Sequencing was carried out on an automated DNA sequencer (ABI PRISM 377 DNA Sequencer; Applied Biosystems).

3. Results and discussion

3.1. Inhibition of topo II decatenation by antileishmanial/anti-topo II drugs

Topo II was partially purified using diethylaminoethyl (DEAE)–cellulose chromatography following a published protocol [4] to ca. three-fold both for Ld-Wt and Ld-As20 (data not shown). By titrating different concentrations of the drug, we have previously reported drug concentrations inhibiting 50% of topo II decatenation (IC_{50}) in nuclear extracts of Ld-Wt and Ld-As20 as 242 μg/mL and 320 μg/mL novobiocin, respectively [2]. Similarly, after titrating varying concentrations of drugs, the IC_{50} values of topo II decatenation in Ld-Wt and Ld-As20 nuclear extract, respectively, were 1 μM and 5 μM for pentamidine [3], 1.5 μM and 4.5 μM for doxorubicin [3] and 1.6 μM for actinomycin D (unpublished data). In all the above cases (except actinomycin D), the IC_{50} for topo II activity in Ld-As20 was observed to be higher compared with Ld-Wt, clearly displaying the differential response of the two enzymes towards different topo II inhibitors. When the inhibitory effect of novobiocin (Fig. 1A, lanes 3 and 8), pentamidine (Fig. 1A, lanes 4 and 9), actinomycin D (Fig. 1A, lanes 5 and 10) and doxorubicin (Fig. 1A, lanes 5 and 11), at the abovementioned IC_{50} values in nuclear extract, were tested on 4 μg of partially purified topo II from Ld-Wt and Ld-As20, ca. 40–45% inhibition of decatenation was observed (Fig. 1B). Potential antileishmanial drugs such as miltefosine and sodium arsenite that are known not to inhibit topo II were found to have no effect on topo II decatenation in Ld-Wt and Ld-As20 (data not shown). As evident, there was differential inhibition of topo II activity even in partially purified enzymes. Although the basal level of topo II activity is always higher in Ld-As20 compared with Ld-Wt, when comparing the inhibitory effects of drug comparisons were made with their respective untreated samples. Thus, for the same protein concentration (4 μg) of Ld-Wt and Ld-As20 tested to obtain 50% inhibition of decatenation activity (derived by comparing the treated samples of sensitive strains with the respective untreated sample, and treated samples of resistant strains with the respective untreated sample), ca. 1.3-fold higher novobiocin (242 μg/mL in Ld-Wt vs. 320 μg/mL in Ld-As20), 5-fold higher pentamidine (1 μM in Ld-Wt vs. 5 μM in Ld-As20) and 3-fold higher doxorubicin (1.5 μM in Ld-Wt vs. 4.5 μM in Ld-As20) was required in Ld-As20 (Fig. 1). The

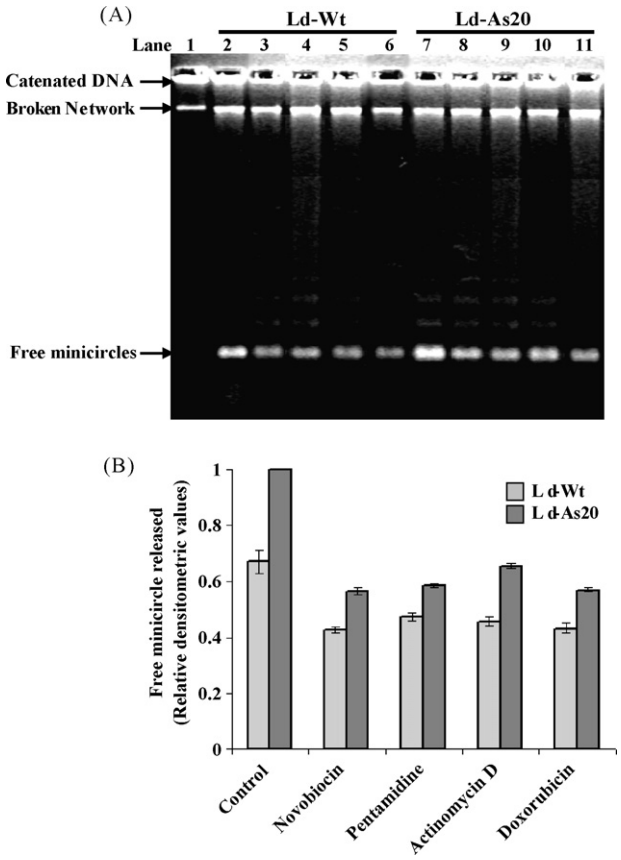

Fig. 1. (A) Inhibition of partially purified topoisomerase II (topo II) activity. Decatenation of 1 μg of kDNA (lane 1) with 4 μg of partially purified topo II from Ld-Wt and Ld-As20 (lanes 2 and 7, respectively) was performed in the presence of 242 μg/mL and 320 μg/mL novobiocin (lane 3 and 8, respectively), 1 μM and 5 μM pentamidine (lanes 4 and 9, respectively), 1.6 μM actinomycin D (lanes 5 and 10) and 1.5 μM and 4.5 μM doxorubicin (lanes 6 and 11, respectively). (B) Densitometric analysis of (A) denoting free minicircle released. Results are expressed as the mean ± standard error of the mean of three independent experiments.