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Short communication

Point mutations in mitochondrial topoisomerase enzymes of *Trypanosoma congolense* are not involved in isometamidium resistance

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Isometamidium chloride (ISM) is a trypanocide widely used in Africa for the treatment or the prevention of cattle trypanosomiasis. This molecule belonging to the family of the phenanthridines has been used in the field for several decades [1] and numerous cases of therapeutic failures have been reported in many countries [2]. The resistant phenotype in some trypanosome field isolates has been confirmed by *in vivo* testing of individual clones derived from the isolates [3–5].

Despite the fact that ISM is already on the market since nearly half a century ago, little is known about the precise mode of action of this compound and about the resistance mechanisms developed by trypanosomes. Delespaux et al. [5] reported the possible role of ABC transporters in ISM resistance but concluded that probably more than one mechanism is involved in resistance to ISM. The aim of this work was to explore a possible alternative pathway of resistance, i.e. the alteration of the proposed targeting site of ISM.

The compartmentalisation of ISM was reported by Wilkes et al. [6] who showed that the trypanosome kinetoplast was the primary site of ISM accumulation. This was later confirmed for the hemoflagellate *Cryptobia salmositica* (*Kinetoplastida* Bodonina) by Ardelli and Woo [7] and for *Trypanosoma brucei* by Boibessot et al. [8] using more sophisticated chromatographical and microscopical techniques. However, Kaminsky et al. [9] showed evidence of toxicity of ISM for dyskinetoplastic strains of *Trypanosoma evansi* and *Trypanosoma equiperdum*, suggesting that the kinetoplast was not the primary site of accumulation of ISM and that ISM might exert its toxic effect elsewhere in the organism of the trypanosome.

In 1990, Shapiro and Englund [10] suggested that diamidine drugs exert their activity within the kinetoplast of *T. equiperdum* which is characteristic of type II topoisomerase inhibitors and that they are mimicking the effects of the antitumor drug etoposide (specific inhibitor of the enzyme topoisomerase II). The disappearance of the kinetoplast DNA network is induced by the use of specific topoisomerase II inhibitors [11] and the same progressive shrinking of the DNA network of the kinetoplast was observed by Wang and Englund [12] when silencing the mitochondrial topoisomerase gene by RNA interference.

Topoisomerase II-targeting drugs are very effective to kill fast dividing cells as it is the case for tumoral cells [13] as well as for trypanosomes or *Leishmania* [14].

Alteration of drug targeting sites is a common phenomenon in cells which develop drug resistance. A mutated topoisomerase I (nuclear) has been observed in *Leishmania donovani* where a Gly 185 Arg and a Asp 325 Glu mutation conferred resistance to camptothecin [15]. Alterations in a topoisomerase II were also correlated with drug resistance in ovarian carcinoma cells [13]. In order to assess whether topoisomerases are involved in resistance to ISM we examined the two genes coding for the potential targeting site of ISM in *Trypanosoma congolense*, the two mitochondrial topoisomerases or type II topoisomerases. In *T. brucei*, the two genes coding for mitochondrial topoisomerases (GeneDB database Tb09.160.4090, TOP2 (3666 bp) and Tb11.01.3390, TOP2 (4275 bp)) are located on chromosomes 9 and 11, respectively. Orthologues of the two genes are found in *Leishmania major*, *Trypanosoma vivax* and *Trypanosoma cruzi* (two haplotypes per gene). Only one annotated ortholog was found in

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T. congolense congo927e11.p1k1 (GeneDB temporary ID) and was renamed in this study T.congoTOPOII$_{(9)}$. *T. congolense* reads were assembled to obtain the open reading frame (ORF) corresponding to the Tb11.01.3390, TOP2 (smallest sum probability 5.6e^{-59}) and named T.congoTOPOII$_{(11)}$. This assembled ORF was checked for consistency through the entire sequencing of the coding sequence (GenBank DQ887563).

The two *T. congolense* genes coding for the two different mitochondrial topoisomerases were thus analysed for polymorphism in order to assess whether they are involved in resistance to ISM. The first mitochondrial topoisomerase gene T.congoTOPOII$_{(9)}$ was screened for point mutations by comparing SSCP profiles of four sensitive (strain number 1–4 in Table 1) and eight resistant strains (strain number 5–12 in Table 1). The second mitochondrial topoisomerase gene T.congoTOPOII$_{(11)}$ was screened for point mutations by the sequencing of the open reading frames of three *T. congolense* strains, one sensitive and two resistant to ISM. The savannah type *T. congolense* IL1180 was used in this study as the sensitive reference strain. IL1180 has a CD50 (the curative dose that gives complete cure in 50% of the animals) in mouse of 0.018 mg/kg [16]. Two other *T. congolense* strains isolated from cattle in Eastern Province of Zambia (JM 158) and Burkina Faso (SA95), resistant to 1 mg/kg ISM in the single dose mouse test [17] were used as resistant reference strains. Since these two strains were identified as sensitive using the PCR-RFLP technique described by Delespaux et al. [5], which detects a mutation in a putative ABC type-like multidrug transporter, it is assumed that they probably use an alternative mechanism of resistance to ISM. Twenty-one other *T. congolense* strains characterized for ISM resistance *in vivo* and by PCR-RFLP [5] were used for further investigation of the sequence differences observed among the T.congoTOPOII$_{(11)}$ genes of the three above-mentioned reference strains (one sensitive, two resistant). Details about the strains used in this study are shown in Table 1. All strains were isolated from cattle, except IL1180 and IL3343 which originated from a lion.

Table 1
Origin of the 24 *Trypanosoma congolense* savannah type isolates used in this study and PCR-RFLP results of the 19 T.congoTOPOII$_{(11)}$ test amplicons

Number	Strain	Taq I	Sensitivity to ISM[a]	Country	Provided by
1	IL1180	R[b]	Sensitive	Tanzania	ILRI[c]
2	TRT 8	U[d]	Sensitive	Zambia	ITM[e]
3	TRT 54	U	Sensitive	Zambia	ITM
4	KTT/Msoro M7	M	Sensitive	Zambia	ITM
5	IL3343	M	Resistant	Tanzania	ILRI
6	SA95R	U	Resistant	Burkina Faso	CIRDES[f]
7	JM158	U	Resistant	Zambia	ITM
8	JM210	M	Resistant	Zambia	ITM
9	TRT57	M	Resistant	Zambia	ITM
10	KONT174	ND	Resistant	Cameroon	ITM
11	PA77	ND	Resistant	Ethiopia	CIRDES
12	KTT/Mphita 4028	ND	Resistant	Zambia	ITM
13	J4J4	U	Sensitive	Zambia	ITM
14	EATRO 1157	U	Sensitive	Uganda	ITM
15	GUTR 28	U	Sensitive	Gambia	ITM
16	KTT/Yobo 2038	M[g]	Sensitive	Zambia	ITM
17	GUTR28	ND	Sensitive	Gambia	ILRAD[h]
18	GUTR37	ND	Sensitive	Gambia	ILRAD
19	SA268	U	Resistant	Burkina Faso	CIRDES
20	Karan	U	Resistant	Burkina Faso	ITM
21	Djuma	M	Resistant	RDC	ITM
22	TRT2	U	Resistant	Zambia	ITM
23	TRT10	U	Resistant	Zambia	ITM
24	TRT25	M	Resistant	Zambia	ITM

[a] Single dose mouse test (1 mg/kg).
[b] Restricted.
[c] International Livestock Research Institute.
[d] Unrestricted.
[e] Institute of Tropical Medicine of Antwerp.
[f] Centre International de Recherche, Développement sur l'Elevage en zone Subhumide.
[g] Mixed profile restricted and unrestricted.
[h] International Laboratory for Research on Animal Diseases.

Cryostabilates of trypanosomes (DMSO 20% as cryopreservative) were reactivated by intraperitoneal injection in mice. At the first peak of parasitaemia, the mice were euthanized, and the blood collected with anticoagulant. The DNA was then extracted using the QIAamp® DNA Blood Midi Kit. The PCR-restriction fragment length polymorphism (PCR-RFLP) technique using the small subunit of the ribosomal DNA (Ssu-rDNA) was used to confirm the savannah type of the *T. congolense* isolates [18].

Seven sets of primers were then designed for the amplification and SSCP analysis of the 3684 bp gene (T.congoTOPOII$_{(9)}$) using the Primer3 [19] computer programme (http://frodo.wi.mit.edu) on the contig sequence congo927e11.p1k1 (GeneDB, Wellcome Trust Sanger Institute), see Table 2 for details.

Two supplementary sets of primers were designed for the amplification and cloning of the putative 4317 bp T.congoTOPOII$_{(11)}$ gene using the same Primer3 computer programme on reads available on GeneDB and assembled in a putative ORF. The first forward primer (ATOPOII$_{(11)}$ F1 AAGATGAAGCGATGGGCTCA) was selected 910 bp upstream the start codon of the topoisomerase II gene, the first reverse primer (ATOPOII$_{(11)}$ R4 CGATGTTGGTGAGTCGCGTAAAG) was selected at bp 2327 after the start codon, the second forward primer (BTOPOII$_{(11)}$ F2 CAAGAAGGATGACGACGATA) at bp 1859 after the start codon and the second reverse primer (BTOPOII$_{(11)}$ R2 TAATGCCAACGTGCTCCCTA) 456 bp downstream the stop codon. The expected sizes of the two PCR products were 3260 and 2917 bp for the first and the second, respectively. One extra set of primers was designed to amplify the region of the T.congoTOPOII$_{(11)}$ gene spanning the single nucleotide polymorphism (SNP) for further characterization by RFLP using the Primer3 [19] computer programme. The primer sequences are TOPOII$_{(11)}$ 3872F TCCGAAGACTTCAAGAGCAAAG and TOPOII$_{(11)}$ 3872R CAGGGGCCTCTATGAAACATATT.

For the SSCP analysis of the T.congoTOPOII$_{(9)}$ gene, standard PCR amplifications were carried out in 25 μl reaction mixtures containing 5 μl DNA sample (at 10 ng μl^{-1} in case of reference DNA samples), 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 20 pmol of each primer (see Table 2), 0.5 U *Taq* polymerase enzyme (Goldstar, Eurogentec). The reaction mixture was overlaid by 50 μl fine neutral mineral oil (Sigma) and placed on a heating block of

Table 2
Size of the seven amplicons used in the SSCP analysis of the T.congoTOPOII$_{(9)}$ gene, restriction enzymes and primers sequences

Amplicon	Size (bp)	Restriction enzymes	Forward primer	Reverse primer
TOPOII$_{(9)}$ 1	577	*Mbo*II–*Rsa*I	CAAGAAGCTTACCCCCATTGAGCAT	TCGCTCGTAGTCAGGAAGGAACTTCA
TOPOII$_{(9)}$ 2	608	*Hha*I–*Hph*I	GCGTTTCGAACGTTGGGTCAAAAG	GGGCATCCGTAGAAGGTATTTCATCAC
TOPOII$_{(9)}$ 3	614	*Hha*I–*Rsa*I	CCGACTTATTTCCGCACCCACAA	TCACGCAGACGCGCCTTTACAAT
TOPOII$_{(9)}$ 4	592	*Alu*I–*Hph*I	CCATATTTTCCACGCCCATTGTAAAGG	CCGCCAGTAAAGTTTTGAGCCATTT
TOPOII$_{(9)}$ 5	611	*Alu*I–*Hph*I	GTGGCTCAGCTTTCGGGTTACATTTC	TAAGACCAAGCTCACTTTCGCACTCG
TOPOII$_{(9)}$ 6	624	*Hin*fI–*Cla*I	CTATTGCAAGGGTAACCGTGTGCATG	AACTGCACGGGCGTTGTCTTCTTTAG
TOPOII$_{(9)}$ 7	450	*Bst*NI–*Cac*8	AGAAGATTGCTGAGACGGAACGTCGT	GAGGCGCGATACCTTGTTAGCGATAGTA

a programmable thermocycler (PTC-100 TM, M.J. Research Inc.). After a denaturation step of 4 min at 94 °C each of the 40 cycles consisted of 30 s at 94 °C, 45 s at 58 °C and 60 s at 72 °C. A 5 μl volume of each sample was electrophoresed in a 2% agarose gel for 20 min and stained with ethidium bromide for 30 min.

For the DNA amplification of the T.congoTOPOII$_{(11)}$ gene, standard PCR amplifications were carried out as previously mentioned but with a longer extension time of 3 min and an electrophoresis in a 1% agarose gel because of the long size of the amplicons.

For the sequencing of the T.congoTOPOII$_{(11)}$ genes of the three reference strains (sensitive IL1180 and resistant JM158 and SA95R), the two PCR products of 3260 and 2917 bp were cloned using the Topo-cloning® kit (Invitrogen, Carlsbad, CA, USA), exactly as described by the manufacturer. The recombinant plasmids containing the desired inserts were purified and their inserts sequenced using the Model 377-XL Sequencer (PE-Applied Biosystems, Eurogentec®, Belgium). The TOPOII$_{(11)}$ test amplicon obtained from the SNP-subregion was used to analyse the field strains which were previously characterized for ISM resistance with the single dose mouse test [11]. PCR conditions were as described for the amplicons of the SSCP analysis of the T.congoTOPOII$_{(9)}$ gene but with the TOPOII$_{(11)}$ 3872F and TOPOII$_{(11)}$ 3872R as primers.

A specific restriction enzyme was chosen using the clustal alignment of the sequences of the TOPOII$_{(11)}$ test amplicon of the three above-mentioned reference strains (one sensitive, two resistant). Digestions of the TOPOII$_{(11)}$ test amplicons were performed with *Taq I* in NEBuffer 3 (New England BioLabs) according to the manufacturer's specifications (MBI Fermentas, Lithuania). Four microlitres of digested sample mixed with 2 μl loading buffer were transferred onto a 10% polyacrylamide gel together with a 100 bp DNA ladder (MBI Fermentas, Lithuania) for fragment size determination. DNA fragments were separated by electrophoresis in 0.5× TBE buffer at 100 V for 2.5 h. The gel was stained using a commercial silver staining kit (Silver staining kit DNA plusone, Pharmacia Biotech, Uppsala, Sweden) and mounted for storage.

For the SSCP analysis of the T.congoTOPOII$_{(9)}$ gene, restriction enzymes were chosen to cut the amplicons (between 450 and 624 bp) in fragments of an adequate size to allow optimal SSCP analyse (optimal range between 100 and 300 bp). Digestions were performed with the restriction enzymes mentioned in Table 2 (two restriction enzymes with distinct recognition sites per amplicon) in the appropriate NEBuffer (New England BioLabs) according to the manufacturer's specifications, using 10 U μg^{-1} DNA (0.6 U μl^{-1} PCR product) on 6 μl of amplified DNA in 15 μl total volume. The reaction was left overnight in a water bath at the specified temperature.

The electrophoresis and staining for the SSCP analysis of the T.congoTOPOII$_{(9)}$ gene, was performed as follows: 8 μl of D2 buffer (95% formamide, 10 mM NaOH, 0.05% bromophenol blue, 0.05% xylene cyanol) was mixed with 4 μl of PCR product then heated for 5 min at 95 °C, cooled on ice and left for at least 10 min at minus 18 °C. Six microlitres of this mixture was added on an ELCHROM GMA gel©. The electrophoresis was run at 8 °C and the gels were stained with SYBR green according to the manufacturer's specifications.Our results of the SSCP analysis of the TOPOII$_{(9)}$ gene show the conserved character of the gene and did not reveal any polymorphism related to drug resistance. In the same way, the analysis of the predicted amino acid sequence of the fully-sequenced T.congoTOPOII$_{(11)}$ genes of the three reference strains showed a single Val 1309 Gly mutation in the two resistant strains. Some other base changes were observed but did not affect the amino acid sequences of the translated sequences and were thus not further investigated. This Val 1309 Gly mutation could not be further correlated with the resistance phenotype in the PCR-RFLP analysis. The TOPOII$_{(11)}$ test amplicon gave a profile with a single band of 470 bp for the unrestricted products and a profile with a 416 and a 54 bp bands for the restricted amplicons (*Taq I*). Results summarised in Table 1 indicate that the sequence difference observed between the sensitive and resistant reference strains cannot be extrapolated to other strains with known sensitivity or resistance profile to ISM.

The aim of this work was to explore possible alterations of the proposed targeting site of ISM. Our results show that the two *T. congolense* genes coding for a topoisomerase II enzyme are highly conserved in the strains characterized in this study. When considering the vast geographical distribution of the strains (Table 1) originating from the tsetse fly belt in East and West Africa, it is very likely that the conserved nature of the topoisomerase II in trypanosomes is the rule. The SSCP technique is assumed to be able to detect 90% of the potential base changes [20]. It is well known that base changes at the extremities of the fragments are very difficult to detect because of the minimal influence on the three-dimensional structure of the single strand DNA. To alleviate this problem or at least to increase the sensitivity of the technique, the amplicons used for the SSCP analysis of the T.congoTOPOII$_{(9)}$ gene were digested with two

different restriction enzymes in order to get the base changes more centrally in the fragment. The highly conserved nature of the T.congoTOPOII$_{(9)}$ gene and the fact that the reads for T.congo TOPOII$_{(11)}$ were not assembled yet, led us to undertake sequencing, a more sensitive method but far more expensive for the T.congoTOPOII$_{(11)}$ gene. It allowed us to confirm the highly conserved character of the latter gene. Taking into account the low probability that the SSCP technique would have failed to detect some strategic point mutations and the fact that in other trypanosome species as well as in *Leishmania*, there are consistently two distinct mitochondrial topoisomerases and not more, it seems that contrary to what is found in *L. donovani* and in certain cancer cells, changes in the topoisomerase II genes do not appear not to be a strategy followed by *T. congolense* to develop resistance to phenanthridines.

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