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The susceptibility of *Trypanosoma congolense* and *Trypanosoma brucei* to isometamidium chloride and its synthetic impurities

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ABSTRACT

Since the 1950s, the chemotherapy of animal African trypanosomosis in cattle has essentially relied on only two compounds: isometamidium chloride (ISM), a phenanthridine, and diminazene aceturate, an aromatic diamidine. The commercial formulations of ISM, including Veridium® and Samorin®, are a mixture of different compounds: ISM is the major component, mixed with the red isomer, blue isomer and disubstituted compound. To investigate the pharmacological effects of these individual compounds ISM, the blue and red isomers and the disubstituted compound were synthesised and purified by HPLC. The activity of each compound was analysed both *in vitro*, and in mice *in vivo*. For the *in vitro* analysis, a drug sensitivity assay was developed in 96-well tissue culture plates to determine the effective concentration which killed 50% of trypanosome population within 48 h of drug exposure (IC_{50}). All compounds tested *in vitro* possessed trypanocidal activity, and purified ISM was the most active. Veridium® and Samorin® had similar IC_{50} values to purified ISM for both *Trypanosoma congolense* and *Trypanosoma brucei brucei*. The disubstituted compound had the highest IC_{50} values whereas intermediate IC_{50} values were obtained for the blue and red isomers. *In vivo*, single-dose tests were used to evaluate the trypanocidal and prophylactic activity against *T. congolense*. Interestingly, the prophylactic effect two months post treatment was as efficient with ISM, Veridium®, Samorin® and the disubstituted compound at the highest dose of 1 mg/kg whereas the red and blue isomers both showed much lower prophylactic activity. This study on *T. congolense* implies that it is necessary to limit the quantity of the blue and red isomers in the commercial mixture. Finally, the *in vitro* sensitivity assay may be useful for screening new trypanocides but also for the testing and detection of resistant trypanosome isolates.

1. Introduction

African trypanosomosis is a parasitic disease caused by flagellated protozoa of the order of Kinetoplastidae and genus *Trypanosoma*. Trypanosomes are transmitted to mammals by tsetse flies and are responsible for the diseases *Nagana* in cattle and sleeping sickness in humans. The

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pathogenic agents for animal African trypanosomosis in cattle are *Trypanosoma congolense*, *Trypanosoma vivax*, and to a lesser extent, *Trypanosoma brucei brucei*. No vaccine is available, thus chemotherapy remains the most commonly employed method to control trypanosomosis.

Among available drugs for animal trypanosomosis, diminazene aceturate is used therapeutically, and isometamidium chloride (ISM) is used both therapeutically and prophylactically. Despite the fact that ISM has been on the market for more than 50 years, very little is known about the precise mode of action of this compound. It has previously been shown that ISM is associated with the kinetoplast in *T. congolense* and *T. b. brucei* (Boibessot et al., 2002; Wilkes et al., 1997) and that the mitochondrial electrical potential was responsible for the ISM uptake in *T. congolense*. However, other targets must also exist, since some dyskinetoplastic strains of *Trypanosoma evansi* and *Trypanosoma equiperdum* are sensitive to ISM (Kaminsky et al., 1997).

The synthesis of the commercial form of ISM (including Veridium® and Samorin®) results in a mixture of compounds including: isometamidium [8-(3-*m*-amidinophenyl-2-triazeno)-3-amino-5-ethyl-6-phenylphenanthridinium chloride hydrochloride, M&B4180A], the red isomer [3-(3-*m*-amidinophenyl-2-triazeno)-8-amino-5-ethyl-6-phenylphenanthridinium chloride hydrochloride, M&B38897], blue isomer [7-(*m*-amidinophenyldiazo)-3,8-diamino-5-ethyl-6-phenylphenanthridinium chloride hydrochloride, M&B4250] and disubstituted compound [3,8-di(3-*m*-amidinophenyltriazeno)-5-ethyl-6-phenylphenanthridinium chloride dihydrochloride, M&B4596] (Fig. 1). Although the quantity of each compound differs between the commercial products, it has been shown that ISM is always the major component and the disubstituted compound is the least abundant (Schad et al., 2008).

Some limited studies with chemically synthesised compounds have previously endeavoured to identify the effect of these, and other phenanthridine compounds against trypanosomes (Brown et al., 1961). However, the limitations of the purification and analytical methods available at the time made it difficult to obtain pure compounds, thus the data collected on the pharmacological effects of each individual compound is uncertain (Kinabo and Bogan, 1988). Determining the effects of the purified individual compounds is necessary in order to understand the efficacy, bioavailability and toxicological effects of the commercial formulations of ISM.

In the current study, the efficacy of three major by-products of ISM synthesis was compared to purified ISM, as well as the commercial products, Veridium® and Samorin®. For the first time, the red and blue isomers and the disubstituted compound were synthesised, purified by HPLC and tested individually for trypanocidal activity against *T. congolense* and *T. b. brucei in vitro*, in addition to analysis of trypanocidal and prophylactic activity against *T. congolense in vivo*. Analysis of trypanocidal activity *in vitro* necessitated the development of a 96-well sensitivity test to determine IC_{50} values of the individual compounds.

2. Materials and methods

2.1. Trypanosome strains and culture conditions

T. congolense IL1180, *T. congolense* IL3000 (kindly provided by the International Livestock Research Institute, Nairobi, Kenya) and *T. b. brucei* Antat 1.1 strains were used for this study. *T. congolense* IL3000 and *T. b. brucei* Antat 1.1 bloodstream forms were used since the cultures of these strains are standardised (Baltz et al., 1985; Coustou et al., 2010). *T. congolense* IL1180 was used for *in vivo* sensitivity tests since it causes a chronic infection in mice (Coustou et al., 2010) and was previously used as a reference drug-sensitive strain for Samorin® uptake studies (Peregrine et al., 1988) since it is ISM sensitive (Hirumi, 1993).

2.2. Drugs

Isometamidium (M&B 4180A), the blue isomer (M&B 4250), red isomer (M&B 38897) and disubstituted compound (M&B 4596) were synthesised and purified by Provence Technologies SAS (Hôtel Technologique-BP100, Technopôle de Château-Gombert, 13382 Marseille Cedex 13-France). The compounds were analysed, and structures confirmed by LC/MS (M^+) and NMR (^1H and ^{13}C). Respective purities were 99.7%, 97.6%, 95.4% and 97.9% for ISM, the blue isomer, red isomer and disubstituted compound. Veridium®, Samorin® and the compounds were dissolved in distilled water (10 mg/ml) and stored at −20 °C. For *in vitro* drug sensitivity tests, drugs were subsequently diluted in the trypanosome culture medium.

2.3. In vitro drug sensitivity tests

Prior to the drug sensitivity tests, observation of the general growth patterns of *T. congolense* IL3000 and *T. b. brucei* Antat 1.1 in 96-well plates for 72 h established that an inoculum of 4000 cells was optimal for exponential growth for 48 h. Parasites, counted using a haemocytometer (4000/well, 100 μl), were added to 96-well plates and incubated at 34 °C (*T. congolense*) or 37 °C (*T. b. brucei*) with 4% CO_2 for ∼4 h before addition of drugs. The drugs were initially dissolved in water (10 mg/ml) and serial dilutions made in culture media (1:10). Each dilution of drug (100 μl) was added to 100 μl of culture in wells (duplicates were made for each dilution from 1 mg/ml until 1.10^{-19} mg/ml). Subsequently, parasites were cultivated under the aforementioned conditions for 24 and 48 h. The number of viable cells in culture was quantified using the CellTiter-Glo® Luminescent Cell Viability assay (Promega) according to the manufacturer's instructions. Briefly, CellTiter-Glo® (100 μl) substrate was added to each well, plates were placed on an orbital shaker to induce lysis and subsequently incubated at room temperature (10 min). Luminescent signal was measured using the Fluostar Optima at 405 nm. The background value (medium only) was subtracted. The quantity of live cells was calculated as a percentage of the control (cells without drug). The half-maximal inhibitory concentrations (IC_{50}) were calculated using Sigmaplot12.5 (Systat Software, Inc.). Two independent experiments were conducted for each strain.

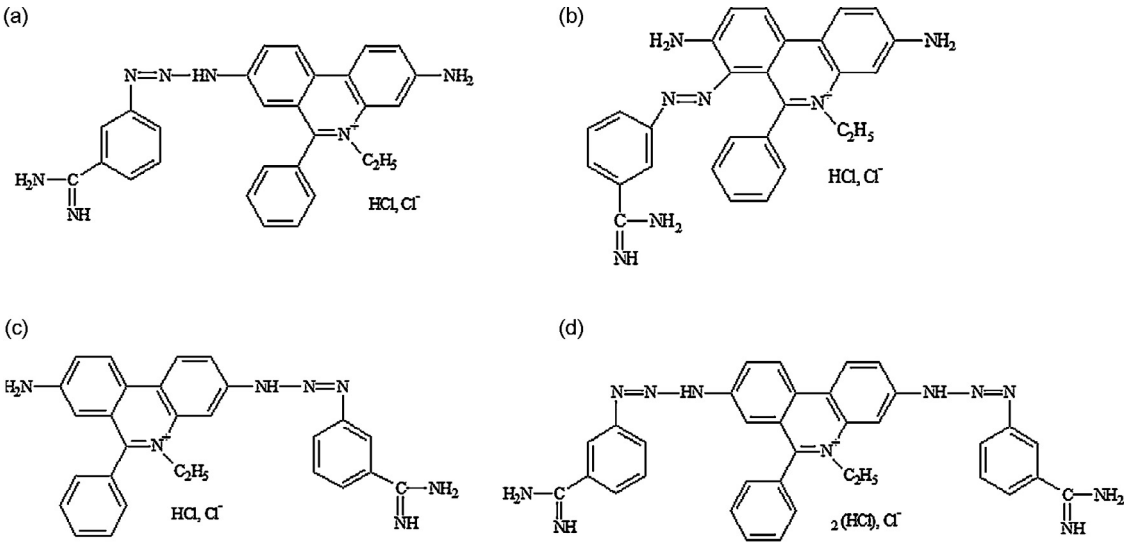

Fig. 1. Chemical structures of isometamidium and its synthetic impurities. (A) Isometamidium (M&B 4180 A); (B) Blue isomer (M&B 4250); (C) Red isomer (M&B 38897); (D) Disubstituted compound (M&B 4596).

2.4. In vivo drug sensitivity tests

All animal procedures were carried out in strict accordance with the French legislation (Rural Code articles L 214-1 to L 214-122 and associated penal consequences) and European Union (2010/63/EU) guidelines for the care of laboratory animals and were approved by the Ethical Committee of Bordeaux (C2EA-50) and by the University of Bordeaux 2 animal care and use committee. All efforts were made to minimise animal suffering.

Female Swiss mice (CD1) were purchased from Charles River (L'Arbresle, France) and used for experiments at eight weeks of age. A single-dose test was used to evaluate the trypanocidal activity of the drugs (Eisler et al., 2001). Briefly, groups of five mice (two for non-treated control groups) were infected by intraperitoneal (i.p.) inoculation with 10^5 trypanosomes (*T. congolense* IL1180) diluted in 200 μl phosphate-buffered saline-glucose (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4, 1% glucose). Mice were treated 24 h after infection. Each drug was administered by i.p. inoculation at concentrations of either 1 mg/kg or 0.1 mg/kg of body-weight. Given the similar results obtained for Samorin® and Veridium® at 1 mg/kg, only Veridium® was tested at the lower dose (0.1 mg/kg). Non-treated control mice only received sterile water (100 μl) post infection. Parasitaemia was monitored microscopically from mouse tail blood twice weekly, and based on the observation of at least 10 fields at 400× magnification. To evaluate the prophylactic effect, mice that were parasite negative after one month were re-infected with 10^5 trypanosomes. The re-infection was repeated after the second and third months post the initial infection and treatment if mice were consistently parasite negative. Parasites for the re-infections were obtained from chronically infected control mice in order to avoid immediate immune clearance due to the presence of homologous anti-VSG antibodies. Two non-treated mice were infected at each infection and re-infection point. Treatment was considered inefficient when more than 2 out of 5 mice within a group were positive for parasites.

3. Results

3.1. In vitro sensitivity of T. congolense and T. b. brucei to Veridium®, Samorin®, isometamidium, red isomer, blue isomer, and the disubstituted compound

Veridium®, Samorin®, purified ISM, the red and blue isomers and the disubstituted compound, have been tested individually for *in vitro* efficacy against *T. congolense* (Fig. 2a) and *T. brucei* (Fig. 2b) at 24 (not shown) and 48 hs of drug exposure. Parasite viability and IC_{50} values (Table 1) were calculated. All the compounds displayed varied levels of trypanocidal activity against both *T. congolense* and *T. b. brucei*.

Isometamidium (IC_{50} 0.56 ± 0.05 ng/ml) displayed comparable trypanocidal activity to Veridium® (0.82 ± 0.25 ng/ml) and Samorin® (IC_{50} 1.75 ± 0.50 ng/ml)

Table 1
IC_{50} values from *in vitro* sensitivity tests after 48 h of drug exposure.

	Mean IC_{50} (ng/ml) ± SD[a]	
	T. congolense IL3000	*T. b. brucei* Antat 1.1
Isometamidium	0.56 ± 0.05	9.24 ± 2.13
Red isomer	3.63 ± 0.55	202.15 ± 62.92
Blue isomer	7.11 ± 0.76	12.01 ± 2.22
Disubstituted compound	66.27 ± 14.37	>1000
Veridium®	0.82 ± 0.25	11.06 ± 3.02
Samorin®	1.75 ± 0.50	11.78 ± 4.88

[a] IC_{50} values were obtained by calculation with Sigmaplot 12. Data represent the mean and standard deviation of two separate experiments with two replicates each.

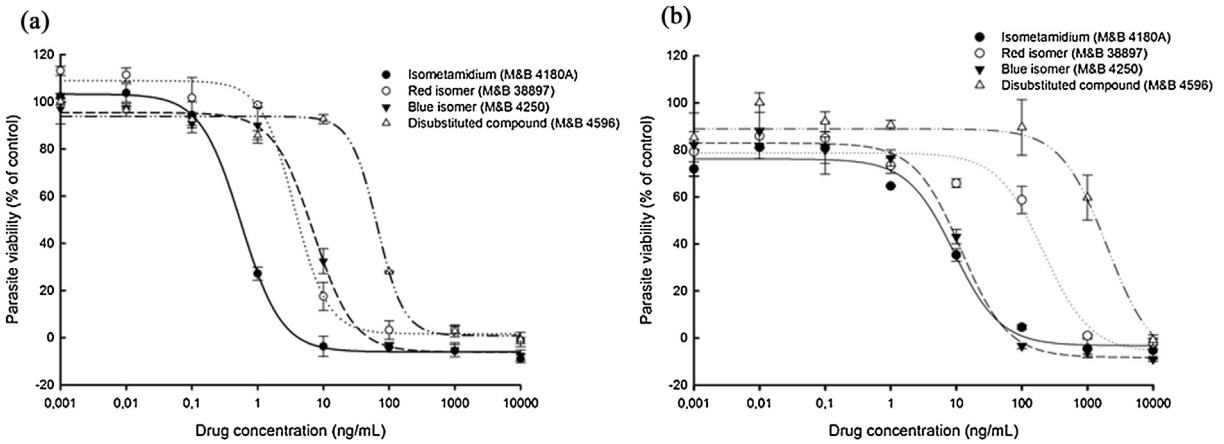

Fig. 2. *In vitro* sensitivity of (A) *Trypanosoma congolense* IL3000 and (B) *T. b. brucei* Antat 1.1 bloodstream form parasites to isometamidium and its derivatives after 48 h of drug exposure. *T. congolense* or *T. b. brucei* cells (4000) were added to 96-well tissue culture plates. After 4 h at 34 °C, drugs were added to cells for 48 h. The number of viable cells in culture was determined by quantification of the ATP present in culture medium with the CellTiter-Glo® Luminescent Cell Viability assay.

against *T. congolense.* The blue and red isomers (IC_{50} 7.11 ± 0.76 ng/ml and 3.63 ± 0.55 ng/ml respectively) exhibited similar trypanocidal activities, but both were ten times less effective than Veridium® and Samorin®. The disubstituted compound was the least potent trypanocide (IC_{50} 66.27 ± 14.37 ng/ml).

For *T. b. brucei*, ISM and the blue isomer (IC_{50} 9.24 ± 2.13, 12.01 ± 2.22 ng/ml respectively) had comparable activity to Veridium® (11.06 ± 3.02 ng/ml) and Samorin® (IC_{50} 11.78 ± 4.88 ng/ml). Similar to *T. congolense*, the red isomer was 10 times less effective (IC_{50} 202.15 ± 62.92 ng/ml) and the disubstituted compound 100 times less potent than ISM respectively (IC_{50} > 1000 ng/ml).

3.2. *In vivo sensitivity of T. congolense to Veridium®, Samorin®, ISM, red isomer, blue isomer, disubstituted compound*

The trypanocidal and prophylactic activity of Veridium®, Samorin®, purified ISM and the red and blue isomers and disubstituted compound were individually tested *in vivo* in mice by monitoring the survival rate after four infections with 10^5 *T. congolense* IL1180 parasites (Table 2). The first infection 24 h before treatment assessed trypanocidal activity, whereas the subsequent challenges gave an indication of prophylactic activity. All the compounds, except the disubstituted compound at a dose of 0.1 mg/kg, protected mice from the initial infection 24 h post-treatment. Samorin®, Veridium® and ISM proved to be very similar in terms of prophylactic activity *in vivo*, protecting mice from two challenges, the last being two months post treatment. The disubstituted isomer, while showing no trypanocidal activity at a dose of 0.1 mg/kg, displayed similar prophylactic activity to Samorin® and Veridium® at the higher dose of 1 mg/kg. The blue isomer did not show any prophylactic effect at either of the tested doses whereas the red isomer showed partial prophylactic activity at the highest dose, one month post treatment.

4. Discussion

In the present study, the efficacy of the commercial products Veridium® and Samorin® were compared to pure ISM, and its synthetic by-products, the red and blue isomers and the disubstituted compound (Tettey et al., 1999). Trypanocidal activity was measured *in vitro* and *in vivo* and prophylactic activity tested by survival of mice *in vivo.*

To test the trypanocidal properties of these compounds *in vitro*, a new drug sensitivity test in 96-well tissue culture plates was developed which will be very useful for rapid screening of new trypanocides, or for any general assays of inhibitors or growth promoting factors. Although laboratory tools for the detection of *in vitro* drug sensitivity have been described previously (Delespaux et al., 2008; Gray and Peregrine, 1993; Hirumi, 1993), the technique proposed in this paper is simple and the least time-consuming.

ISM, Veridium® and Samorin® all showed similar trypanocidal activity *in vitro* against *T. b. brucei* and *T. congolense.* Significantly, the by-products displayed lower trypanocidal activities than pure ISM, since the two isomers and the disubstituted compound had IC_{50} values approximately 10-fold and 118-fold higher than ISM respectively against *T. congolense*. For this reason, the presence of these by-products at high quantities (the red and blue isomers may together constitute up to 40% of the final product, (Schad et al., 2008) in commercial preparations of ISM is concerning given the prevalence of ISM-resistant *T. congolense* strains (Delespaux et al., 2008). Interestingly, the *in vitro* results indicated that *T. b. brucei* was 15-fold less sensitive to ISM than *T. congolense*. Since it is known that a difference in mitochondrial energy metabolism existing among trypanosomatids (Tielens and van Hellemond, 2009); and that the mitochondrial electrical potential may play a role in ISM uptake (Wilkes et al., 1997); this could explain the different level of sensitivity between *T. congolense* and *T. b. brucei.*

Two different doses of the compounds (0.1 and 1 mg/kg) were used for the *in vivo* studies to approximate the range of the doses used in the field (Diarra et al., 1998), either

Table 2
In vivo sensitivity of *T. congolense* IL1180 to isometamidium and its derivatives at two different doses.

Drug	Dose (mg/kg)	Number of parasite positive mice/total Number of successive infections[a]			
		1	2	3	4
Samorin®	1	0/5	0/5	1/5	5/5
Veridium®	0.1	0/5	0/5	0/4[b]	4/4[b]
	1	0/5	0/5	1/5	5/5
Isometamidium	0.1	0/5	0/5	0/5	5/5
	1	0/5	0/5	0/5	5/5
Red isomer	0.1	0/5	5/5	ND[c]	ND
	1	0/5	1/5	5/5	ND
Blue isomer	0.1	0/5	5/5	ND	ND
	1	0/5	4/5	5/5	ND
Disubstituted	0.1	5/5	ND	ND	ND
compound	1	0/5	0/5	1/5	4/5
Non-treated[d]	–	2/2	2/2	2/2	2/2

[a] Mice were infected with 10^5 parasites and treated 24 h later to study the trypanocidal effect. Mice which were negative for parasites after the first month, and each subsequent month were re-infected to establish the duration of prophylactic activity.
[b] One mouse died following the second infection changing the group total to 4.
[c] Not done: treatment was considered inefficient if more than 2 mice per group were infected.
[d] Two mice were infected at each infection and re-infection point as controls.

for trypanocidal treatment or prophylaxis, which also differs depending on the sensitivity of the strain (Gray et al., 1993; Peregrine et al., 1988; Wilkes et al., 1997). Since the commercial products may contain as little as 6% of some of the by-products (disubstituted compound), the lower dose (0.1 mg/kg) is more representative of dose rates achieved for the by-products under field conditions when animals are dosed at 0.5–1 mg/kg with the commercial mixtures.

In terms of trypanocidal effect, the *in vivo* results at the doses tested with the ISM-sensitive strain, confirmed the *in vitro* tests to the extent that all the compounds tested were active except for the disubstituted compound at a lower concentration The disubstituted compound had an IC_{50} 118-fold higher than ISM *in vitro*, therefore, it was not surprisingly that higher dose would be required for a similar level of trypanocidal activity to ISM *in vivo*.

ISM, Veridium®and Samorin® and the disubstituted compound (at 1 mg/kg only) showed similar prophylactic activities against *T. congolense* challenge in mice *in vivo*. This prophylactic activity achieved at 1 mg/kg with the disubstituted compound could be explained by the fact that the disubstituted compound acts like a pro-drug and may be cleaved *in vivo* to produce ISM. Although the disubstituted compound was known to be prophylactic (Brown et al., 1961), the current study demonstrated that this activity is highly dose-dependent. For this reason, it is crucial to note that a standard dose of commercial ISM products contains less than 0.1 mg/kg of the disubstituted compound (Schad et al., 2008), which would be insufficient for a trypanocidal effect. Furthermore, the red and blue isomers, the most abundant by-products (40%) of the commercial products (Schad et al., 2008), have lower prophylactic activities providing only partial protection at one month post treatment.

It is known that qualitative and quantitative differences exist between the different commercial forms of ISM which may arise due to a variety of factors, including temperature and pH, during the manufacturing process (Berg, 1963). This study has demonstrated that these variations are of serious concern since underdosing of ISM or a competition between the red or blue isomers with ISM could increase the risk for the development of drug resistance. Furthermore, the *in vivo* and *in vitro* results with *T. congolense* indicated that the red and blue isomers, the most abundant compounds in synthesis other than ISM, have poor trypanocidal and prophylactic activity. For these reasons, it is essential to establish guidelines to modulate the quantity of the various by-products in the final mixture of the commercial product. This could be achieved by altering the conditions during the manufacturing process. For example, it is known that a pH of 1.8–2.2 in the reaction medium correlates with an increase in the quantity of ISM, whereas a higher pH results in a greater production of the blue isomer (Berg, 1963; Tettey et al., 1999). Thus, optimisation of the manufacturing process, and the strict adherence thereof, would be necessary to control the quantities of the by-products.

The data generated by this study will be very useful to improve the understanding of the efficacy and bioavailability or biotransformation of the commercial formulations of ISM and help to develop new drug compositions with optimal ratios for an improved trypanocidal and prophylactic effects.

Conflict of interest statement

The authors declare that no competing interests exist.

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