The individual components of commercial isometamidium do not possess stronger trypanocidal activity than the mixture, nor bypass isometamidium resistance

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\textbf{A R T I C L E  I N F O} \\
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\textbf{A B S T R A C T} \\
The four components present in the trypanocidal treatment Samorin, the commercially available formulation of isometamidium, were separated and purified by column chromatography. These compounds as well as the Samorin mixture and the other phenanthridine trypanocide, homidium, were tested on Trypanosoma congolense and wild type, diamidine- and isometamidium-resistant Trypanosoma brucei brucei strains using an Alamar blue drug sensitivity assay. \(EC_{50}\) values obtained suggest that M&B4180A (2) was the most active of the components, followed by M&B38897 (1) in all the strains tested, whereas M&B4596 (4) was inactive. Samorin was found to be significantly more active than any of the individual components alone, against \textit{T. congolense} and all three \textit{T. b. brucei} strains. Samorin and all its active constituents displayed reduced activity against the previously characterised isometamidium-resistant strain ISMR1.

1. Introduction

\textit{Trypanosoma brucei brucei}, \textit{T. congolense} and \textit{T. vivax} are responsible for African animal trypanosomiasis (AAT) or nagana in cattle (Giordani et al., 2016). Control of these tsetse-fly transmitted animal trypanosomes in sub-Saharan Africa, in the absence of a vector control programme or an effective vaccine, depends solely on chemotherapy using isometamidium (ISM), dimazene and ethidium bromide. If ethidium bromide is withdrawn from use due to its established mutagenic properties (Sayas et al., 2015), ISM and dimazene would be the only trypanocides left for prophylaxis and treatment of veterinary African trypanosomiasis respectively (Delespaux and De Koning, 2007). Resistance to ISM in this situation would therefore threaten the control of animal trypanosomiasis. Resistance to ISM in the field has been confirmed by a number of reports (Jamal et al., 2005; Mongube et al., 2012; Moti et al., 2012; Tihon et al., 2017). Hence, the management of AAT requires both the introduction of new drugs and optimization of available drugs to increase treatment options and reduce the risk of resistance. Optimization of available drugs would involve the determination of their mechanism of action and resistance to understand how best to use the drugs and reduce the incidence of resistance.

Resistance to ISM involves a reduction in uptake and/or loss of mitochondrial potential in \textit{T. congolense} (Sutherland et al., 1992; Wilkes et al., 1997), the latter of which was linked, in \textit{T. b. brucei}, to mutation of the \(F_1F_0\) ATPase complex (Eze et al., 2016).

ISM is available commercially as the impure mixture Samorin. The four components which the drug is suspected to contain have been purified to homogeneity, and their structures have been rigorously determined by a combination of NMR and mass-spectrometric techniques (Igoli et al., 2014). For the first time, this analysis showed that the Samorin components exist as reversible di-cationic chloride salts or a dihydro form, with a hydrogen addition to C-6 as depicted in Fig. 1, depending on solvent and pH. The compound 8-(3-m-amidinophenyl-2-triazeno)-3-amino-5-ethyl-6-phenylphenanthridinium chloride hydrochloride (2, M&B4180A) is believed to be the principal active component (Schad et al., 2008), and this is usually the structure for isometamidium given in the literature. The other components isolated, usually dismissed as impurities, were 3-(3-m-amidinophenyl-2-triazeno)-8-amino-5-ethyl-6-phenylphenanthridinium chloride hydrochloride (1, M&B38897, ‘red isomer’), 7-(m-amidinophenyldiazo)-3,8-diamino-5-ethyl-6-phenylphenanthridinium chloride hydrochloride (3, M&B4250, ‘blue isomer’) and 3,8-di(3-m-amidinophenyltriazeno)-5-
ethyl-6-phenylphenanthridinium chloride dihydrochloride (4, M&B4596); see Fig. 1. However, the dihydro forms were not observed using NMR with D2O instead of DMSO, suggesting that in aqueous solution the same compounds may be phenanthridinium ions without a C-6 hydrogen, consistent with the belief that this is the biologically active form.

In this study, the compounds (Fig. 1) obtained from the purification of Samorin were tested on wild type Trypanosoma brucei brucei 427 and Trypanosoma congolense IL3000, alongside several T. b. brucei strains, including the diamidine-resistant B48 strain (Bridges et al., 2007) and

Table 1
Overview of Samorin fractions and control drugs against T. congolense and T. b. brucei.

<table>
<thead>
<tr>
<th>compound</th>
<th>T. congolense IL3000</th>
<th>T. brucei s427</th>
<th>T. b. brucei B48</th>
<th>T. b. brucei ISMR1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC_{50} (μM)</td>
<td>n</td>
<td>EC_{50} (μM)</td>
<td>n</td>
</tr>
<tr>
<td>1A</td>
<td>0.035 ± 0.005</td>
<td>5</td>
<td>3.48 ± 0.42</td>
<td>4</td>
</tr>
<tr>
<td>1B</td>
<td>0.65 ± 0.06</td>
<td>5</td>
<td>5.31 ± 0.48</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>0.035 ± 0.005</td>
<td>5</td>
<td>2.67 ± 0.48</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>0.36 ± 0.02</td>
<td>5</td>
<td>12.2 ± 1.4</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>16.0 ± 0.7</td>
<td>6</td>
<td>95.7 ± 17.0</td>
<td>4</td>
</tr>
<tr>
<td>Samorin</td>
<td>0.0033 ± 0.0006</td>
<td>17</td>
<td>0.29 ± 0.04</td>
<td>12</td>
</tr>
<tr>
<td>Ethidium</td>
<td>0.11 ± 0.01</td>
<td>5</td>
<td>3.85 ± 0.22</td>
<td>4</td>
</tr>
<tr>
<td>Pentamidine</td>
<td>0.82 ± 0.02</td>
<td>11</td>
<td>0.0051 ± 0.0004</td>
<td>6</td>
</tr>
</tbody>
</table>

The 50% Effective Concentration (EC_{50}) is given as average and SEM, and the number of replicates (n) for each determination is included in a separate column. RF is the Resistance Factor, the ratio of EC_{50} (s427) and EC_{50} (B48 or ISMR1). The P value is the outcome of an unpaired, two-tailed Student's t-test of the B48 or ISMR1 EC_{50} with the corresponding value of s427.

Fig. 1. Chemical structures of fractions obtained from the fractionation of Samorin. Compounds 1–4 were isolated by Igoli et al. (2014) and matched against their M&B codes. Compounds 1A and 1B are identical (compound 1) but compound 1B contains a significant percentage of ethidium contamination.
ISM-resistant (ISM-R1) strain (Eze et al., 2016). While a report by Sahin et al. (2014) similarly described the effect of individual compounds M&B4180A, M&B4250, B4596 and M&B38897 on trypanosomes, these were separately synthesised rather than purified from commercial Samorin, and the NMR and mass spectra were not made available so their identity and purity could not be verified; the structures were given as phenanthridinium mono-cations. Here we investigate whether purification of Samorin might improve activity and whether the purified compounds all displayed similar activity against T. b. brucei strains resistant to diamidines and phenanthridines, respectively. Absence of cross-resistance for some components would not only point to different mechanisms of action, but highlight a way to bypass isometamidium resistance.

2. Material and methods

2.1. Drugs

Samorin was donated by Mérial, France, while Alamar blue dye was obtained as Resazurin sodium salt from Sigma UK. Compounds 1–4 were fractions from column chromatography purification from Samorin, provided by Professor Graham G. Skellern at the Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow.

2.2. Trypanosome strains and culture

Bloodstream-form of T. b. brucei, strain Lister 427 (s427; MtTat 1.2/ BS221), and its derivatives, B48 (diamidine/arsenical resistant) and ISMR1 (phenanthridine resistant) were maintained in full HMI-9 medium with 10% Fetal Bovine Serum at 37 °C as previously described.

Fig. 2. Bar graphs showing the EC50 values obtained for Trypanosoma congolense IL3000, and wildtype s427, diamidine-resistant (B48) and ISM-resistant (ISM-R1) T. b. brucei strains. EC50 values were determined from alamar blue assays using 105 cells/ml of each cell line. Each bar represents the average of at least three independent assays as indicated in Table 1; error bars are SEM. Statistical significance relative to wild-type T. b. brucei is also included in Table 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
D-glucose (Fisher Scientific), 5.56 g of HEPES (Sigma), 2.2 g of NaHCO3 (Sigma), 1 g penicillin/streptomycin solution (Sigma); storage at 4 °C after sterilization.

2.3. Drug susceptibility assays

Drug susceptibilities were determined for bloodstream-form T. b. brucei, B48 and ISMR1 using the Alamar blue (resazurin) assay as described previously, using 23 doubling dilutions from an initial test compound concentration of 20 μM, and 105 trypanosomes/well; plates were incubated at 37 °C/5% CO2 for 48 h and 24 h before and after the addition of Alamar blue, respectively (Rodenko et al., 2007; Gould et al., 2008). 

3. Results and discussion

It is widely acknowledged that there is an urgent need for new drugs against animal trypanosomiasis; above anything else, this need is driven by the increasing failure of the long-standing, cheap and widely available drugs isometamidium (ISM, Samorin®/Trypanidium®, Merial; Veridium®, Sanofi) and isoniazid (Nimura®/Laprevo®) or isoniazid chloride (Novidium®, Merial). AAT drug resistance has now been documented in at least 21 African countries (Assefa and Shibeshi, 2018), a rapid increase from the reported 17 countries in 2013 (Melaku and Birasa, 2013), and the emerging reality is that animal trypanosomiasis can no longer be effectively treated in many parts of Africa, with dire consequences for livelihoods, economies and food security (Giordani et al., 2016; Assefa and Shibeshi, 2018). As new drugs are at the very least years away from making a meaningful contribution in the field, it is essential that we make the most efficient use of the few licenced drugs available. Here we ask the question whether the purification of (any of) the Samorin components would result in a significantly more potent drug able to overcome current levels of resistance.

The activity of compounds 1–4 against T. b. brucei s427, B48, ISMR1 and T. congolense are presented in Table 1 and Fig. 2. The main observation was that Samorin was found to be consistently and significantly (P < 0.0001) more active than any of the fractions. Compound 2 was found to be consistently the most active of all the Samorin components, followed by the pure compound 1A, while compound 4 was consistently the least active. Samorin was 10.4-fold, 9-fold, 54-fold and 1.9-fold; Table 1), although the reactivity of any one of the pure compounds out of the Samorin mix is not a viable answer to the drug resistance crisis. Given these observations, and the fact that a pure isometamidium would be much more expensive, we would advocate an increased investment in new AAT drug discovery instead.

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References


