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In vitro drug susceptibility of two strains of the wildlife trypanosome, *Trypanosoma copemani*: A comparison with *Trypanosoma cruzi*

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ABSTRACT

Trypanosomes are blood protozoan parasites that are capable of producing illness in the vertebrate host. Within Australia, several native *Trypanosoma* species have been described infecting wildlife. However, only *Trypanosoma copemani* has been associated with pathological lesions in wildlife hosts and more recently has been associated with the drastic decline of the critically endangered woylie (*Bettongia penicillata*). The impact that some trypanosomes have on the health of the vertebrate host has led to the development of numerous drug compounds that could inhibit the growth or kill the parasite. This study investigated and compared the *in vitro* susceptibility of two strains of *T. copemani* (G1 and G2) and one strain of *Trypanosoma cruzi* (10R26) against drugs that are known to show trypanocidal activity (benznidazole, posaconazole, miltefosine and melarsoprol) and against four lead compounds, two fenarimols and two pyridine derivatives (EPL-BS1937, EPL-BS2391, EPL-BS0967, and EPL-BS1246), that have been developed primarily against *T.cruzi*. The *in vitro* cytotoxicity of all drugs against *L6* rat myoblast cells was also assessed. Results showed that both strains of *T. copemani* were more susceptible to all drugs and lead compounds than *T. cruzi*, with all IC50 values in the low and sub-μM range for both species. Melarsoprol and miltefosine exhibited the highest drug activity against both *T. copemani* and *T. cruzi*, but they also showed the highest toxicity in *L6* cells. Interestingly, both fenarimol and pyridine derivative compounds were more active against *T. copemani* and *T. cruzi* than the reference drugs benznidazole and posaconazole. *T. copemani* strains exhibited differences in susceptibility to all drugs demonstrating once again considerable differences in their biological behaviour.

1. Introduction

The genus *Trypanosoma* comprises a large number of species and subspecies that are capable of producing detrimental effects on the host. *T. cruzi* for example, is a protozoan that causes Chagas disease in humans and is an important contributor to heart disease in Latin America (Kirchhoff, 1996). This parasite is able to infect different marsupial species in America and has been shown to produce inflammatory lesions in tissues similar to those seen in human infections (Barr et al., 1991; Carreira et al., 1996). Furthermore, trypanosomes from the "*T. brucei* complex" are pathogenic trypanosomes from Africa that cause sleeping sickness in humans, and nagana in vertebrate animals. Common signs of the infection in humans are swollen lymph nodes, fever, anaemia, oedema, and neurological involvement. Other trypanosomes that are considered non-pathogenic may cause harm when they find a new or naïve vertebrate host. For example, within Australia, the accidental introduction of the exotic *T. lewisi* to Christmas Island is hypothesized to have caused a collapse in the population of the endemic rat *Rattus macleari* to the point of complete extinction (Pickering and Norris, 1996; Wyatt et al., 2008). More recently, a genotype of a native Australian trypanosome, *Trypanosoma copemani* G2, was associated with the rapid and substantial population decline of the critically endangered woylie (*Bettongia penicillata*), which saw 90% of the population crash over 10 years (Botero et al., 2013; Wayne et al., 2013a, 2013b). Although, two genotypes of *T. copemani* have been isolated from the blood of woylies (*T. copemani* G1 and G2), only *T. copemani* G2 has been found infecting several tissues in the woylie and other endangered marsupials such as the southern

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brown bandicoot (*Isoodon obesulus*), and chuditch (*Dasyurus geoffroii*). Intracellular structures suggestive of amastigotes as well as extensive inflammatory cell infiltrates and tissue damage have been found in woylie tissues infected with *T. copemani* G2, thus demonstrating pathogenic potential previously not associated with trypanosomes from wildlife in Australia (Botero et al., 2013). *In vitro* experiments have also confirmed *T. copemani* capability to infect cells (Botero et al., 2016). Both genotypes of *T. copemani* firmly clustered in a monophyletic assemblage with different genotypes of *T. copemani* previously described in the blood of other critically endangered and vulnerable Australian marsupials including Gilbert's potoroos (*Potorous gilbertii*), quokkas (*Setonix brachyurus*) (Austen et al., 2009), and koalas (*Phascolarctos cinereus*) (McInnes et al., 2011). 18SrDNA and g*GAPDH T. copemani* phylogenies that included pathogenic trypanosomes such as *T. cruzi* and *T. brucei* have shown a closer relationship between *T. copemani* and *T. cruzi* compared with *T. brucei* and allied species (Austen et al., 2009; McInnes et al., 2011).

The impact that pathogenic trypanosomes have on the health of the vertebrate host has led to the development of numerous drug compounds that could inhibit or kill the parasite. Benznidazole (N-benzyl-2-nitro-1-imidazole-acetamide) for example, is currently used for the treatment of *T. cruzi* infections. Despite this drug not being completely effective, especially in the chronic stage of the disease (Soeiro and de Castro, 2009; Organization, 2010; Jackson et al., 2010; Batista et al., 2011; Alonso-Padilla and Rodriguez, 2014), it is the main drug therapy available to treat the disease. Posaconazole, an ergosterol biosynthesis inhibitor, has also shown potent *in vitro* and *in vivo* activity against *T. cruzi* (de Figueiredo Diniz et al., 2013). Drugs currently used to treat other trypanosomatid infections such as African trypanosomiasis and leishmaniansis include melarsoprol, eflornithine, miltefosine, and also nifurtimox. (Melarsoprol (2-(4-(4,6-diamino-1,3,5-triazin-2-ylamino)phenyl)-1,3,2-dithiarsolan-4-yl)methanol) is an arsenical drug that has been used against late-stage infections with *T. brucei* subspecies (Denise and Barrett, 2001), and miltefosine (hexadecylphosphocholine) is an alkylphosphocholine that was the first and still the only oral drug that can be used to treat visceral and cutaneous leishmaniasis (Dorlo et al., 2012a, 2012b). Eflornithine (α-difluoromethylornithine) an ornithine decarboxylase inhibitor, has been shown to be active against second stage *T.b. gambiense* (Steverding, 2010), and has been used in conjunction with nifurtimox (E-N-(3-methyl-1,1-dioxo-1,4-thiazinan-4-yl)-1-(5-nitrofuran-2-yl)methanimine) against *T. brucei* (Alirol et al., 2013). Although, all these drugs are the main treatment used to combat these trypanosomatid infections, they are less than ideal due to toxicity, adverse side effects and in some cases lack of efficacy against intracellular parasites (Milord et al., 1992; Castro et al., 2006; Pinazo et al., 2013; Hasslocher-Moreno et al., 2012). Attempts to develop new compounds with potent activity against trypanosomes and low toxicity in mammalian cells has led to the discovery of different ergosterol biosynthesis inhibitor compounds with demonstrated *in vitro* and *in vivo* activity against all *T. brucei* subspecies and *T. cruzi*. For example, inhibition of *T. cruzi* CYP51 (sterol 14α-demethylase) has been shown to affect sterol composition and consequently cause damage to the parasites ultrastructure leading to their death (Lepesheva and Waterman, 2011; Hargrove et al., 2013; Keenan et al., 2013c). Recently developed and optimized lead compounds include the ergosterol biosynthesis inhibitors EPL-BS1937, EPL-BS2391, EPL-BS0967, and EPL-BS1246. All have recently been shown to be non-azole inhibitors of *T. cruzi* CYP51 (Hargrove et al., 2013; Keenan et al., 2013a; Keenan et al., 2013b).

Considering not only the potential pathogenicity of *T. copemani* G2 in the woylie, but also that this parasite has been found infecting other critically endangered and vulnerable Australian marsupials, there is the need to evaluate the *in vitro* susceptibility of *T. copemani* to drugs as first steps towards the understanding of possible ways to ameliorate its impact on threatened populations. Therefore, the aims of this paper are to investigate and compare the *in vitro* susceptibility of *T. copemani* G1 and G2, and *T. cruzi* to reference drugs and compounds currently used against pathogenic trypanosomatids.

2. Materials and methods

2.1. Parasites and cells

T. copemani strains G1 and G2 isolated from the blood of woylies (Botero et al., 2013), and the *T. cruzi* strain 10R26 were grown and maintained as epimastigotes by successive passages every 3 days at 28 °C in RPMI medium containing 10% foetal calf serum (FCS), 5 mg/ml penicillin-streptomycin and 2.5 mg/L haemin. *L6* cells (skeletal myoblast cells) purchased from the American Type Culture Collection were used in the drug toxicity assays. Cells were grown in RPMI medium supplemented with 10% FCS at 37 °C and 5% CO_2.

2.2. Test compounds

Miltefosine and melarsoprol were kindly provided by Dr Vanessa Yardley (London School of Hygiene and Tropical Medicine, UK). Benznidazole tablets (Rochagan - 100 mg) were purchased from Roche (Rio de Janeiro, Brazil). Posaconazole was purchased as an oral suspension (Noxafil Schering Corporation, 40 mg/mL) and isolated from the suspension by dilution with water and centrifugation, followed by extraction and recrystallization from hot i-propyl alcohol (Keenan et al., 2012). Four CYP51 inhibitor lead compounds that have been shown to be inhibitors of *T. cruzi*, incluiding two pyridine derivatives EPL-BS0967 and EPL-BS1246 (PDB1 and PDB2 respectively - also known as UDD and UDO), and two non-azole antifungal fenarimoles EPL-BS1937 and EPL-BS2391 (FN1 and FN2 respectively) were kindly provided by Epichem Pty Ltd (Hargrove et al., 2013; Keenan et al., 2012; Keenan et al., 2013c). Their molecular structures are shown in Fig. 1. Drug compounds were dissolved in dimethyl sulfoxide (DMSO) and stored at 4 °C. Immediately before use, drugs were pre-diluted in RPMI media to the desired concentration. The final DMSO concentration did not exceed 1% (v/v) and had no effect by itself on the proliferation of the parasites.

2.3. In vitro compound activity against trypanosomes

Epimastigotes of *T. copemani* G1 and G2, and *T. cruzi* 10R26 strains in the log phase of growth were diluted in RPMI media to 1×10^6 parasites/ml. 100 μl of parasite suspension (1×10^5 parasites/well) was seeded into 96-well flat-bottom plates (Corning, Corning, N.Y.), and then incubated at 28 °C in a seven-fold dilution series covering a range from 1 μM to 0.004 μM for melarsoprol, and 10 μM–0.013 μM for the remainder of the drugs. All concentration ranges were selected based on initial screenings at 10 and 1 μM that showed percentages of inhibition greater than 50% at 10 or 1 μM. Each drug concentration was evaluated in triplicate. Control wells with only compounds and with only parasites (without compounds) were included. After 48 h of compound exposure, 15 μl of AlamarBlue® (Resazurin-AbD Serotec) was added to each plate allowing for a colour change through metabolic oxidation-reduction by viable trypanosomes. Plates were incubated for an additional 24 h. After this time, absorbance was quantified using a Dynex microplate reader at an excitation wavelength of 570 nm and emission wavelength of 590 nm. The percentage of inhibition was