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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

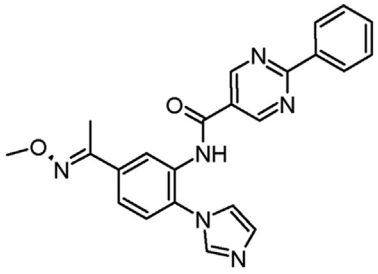
T. copemani strains G1 and G2 isolated from the blood of woylies (Botero et al., 2013), and the *T. cruzi* strain 10R26 were grown and maintained as epimastigotes by successive passages every 3 days at 28 °C in RPMI medium containing 10% foetal calf serum (FCS), 5 mg/ml penicillin-streptomycin and 2.5 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.

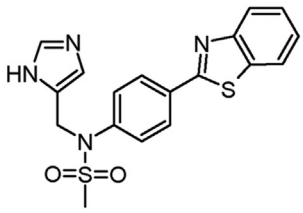
2.3. In vitro compound activity against trypanosomes

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



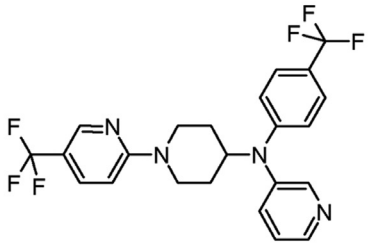
EPL-BS1937 **(FN1)**

N-(2-(1*H*-imidazol-1-yl)-5-[(1E)-*N*-methoxyethanimidoyl]phenyl)-2-phenylpyrimidine-5-carboxamide



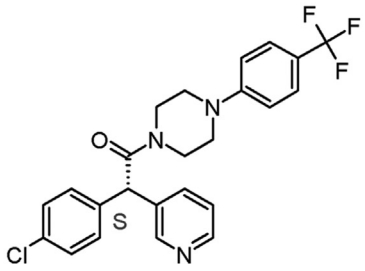
EPL-BS2391 **(FN2)**

N-[4-(1,3-benzothiazol-2-yl)phenyl]-*N*-(1*H*-imidazol-5-ylmethyl)methanesulfonamide



EPL-BS967 **(PDB1)**

N-[4-(trifluoromethyl)phenyl]-N-(1-[5-(trifluoromethyl)pyridin-2-yl]piperidin-4-yl)pyridin-3-amine



EPL-BS1246 **(PDB2)**

(S)-2-(4-Chlorophenyl)-1-{4-[4-(trifluoromethyl)phenyl]piperazin-1-yl}-2-(pyridin-3-yl)ethanone

Fig. 1. Molecular structure of lead compounds.

calculated and used to generate dose-response curves by an average of triplicate data points. The concentration (μM) of the drug necessary to inhibit 50% of cell proliferation of that observed in control cultures (parasites grown in the absence of test compound) was calculated (IC50). Graphs were created and analysed using the statistical software program Prism (GraphPad Software Inc., San Diego, Cali). The statistical significance of results was estimated by 2way ANOVA. Each experiment was performed on three independent occasions.

2.4. In vitro compound toxicity in L6 cells

An evaluation of mammalian cell cytotoxicity was carried out in parallel. 100 μL of RPMI 1640 medium supplemented with 10% foetal bovine serum and containing 5×10^3 *L6* cells were seeded into 96-well plates. Plates were incubated overnight at 37 °C and 5% CO_2 and then drugged with seven 3-fold dilutions covering a range from 10 μM to 0.013 μM for melarsoprol and miltefosine, and 100 μM–0.13 μM for the remainder of the drugs. Control wells with only compounds and with only cells were included. After 72 h of incubation with the drugs, plates were inspected under an inverted microscope to assure growth of cells in the control wells (not drugged) and sterile conditions. 15 μL of AlamarBlue® was then added to wells and the plates incubated for another 2 h at 37 °C and 5% CO_2. Absorbance was quantified using a Dynex microplate reader at an excitation wavelength of 570 nm and emission wavelength of 590 nm. Podophyllotoxin was used as a reference drug for toxicity. The therapeutic index (TI) of all drugs was calculated as TD50/ED50, where TD50 is the dose of drug that causes a toxic response in 50% of the *L6* cells (IC50 value for cytotoxicity) and ED50 is the dose of drug that is active in 50% of trypanosomes (IC50 value for anti-trypanosomal activity). When IC50 values for toxicity were higher than 100 μM, this concentration value was used to calculate the therapeutic index (TI). The statistical significance of results was estimated by 2way ANOVA. Each experiment was performed on three independent occasions.

3. Results

3.1. In vitro compound efficacy of reference drugs

The reduction of resazurin, converted from blue to a bright-red colour by metabolically active trypanosomes/cells, was used as an indicator of viability of trypanosomes and *L6* cells and therefore as a measure of drug activity and toxicity respectively. All reference drugs exhibited potent *in vitro* activity against all trypanosomes. However, both strains of *T. copemani* were more susceptible to all drugs than *T. cruzi.* Benznidazole was approximately eight times more active against *T. copemani* G1 (IC50 1.053 μM) and G2 (IC50 0.713 μM) than against *T. cruzi* (IC50 8.537 μM) (Fig. 2).

Posaconazole exhibited similar activity against *T. cruzi* and *T. copemani* G2, both with an IC50 of 5.429 μM and 6.147 μM respectively. This drug was more active against *T. copemani* G1, which exhibited an IC50 of 1.254 μM. Melarsoprol and miltefosine

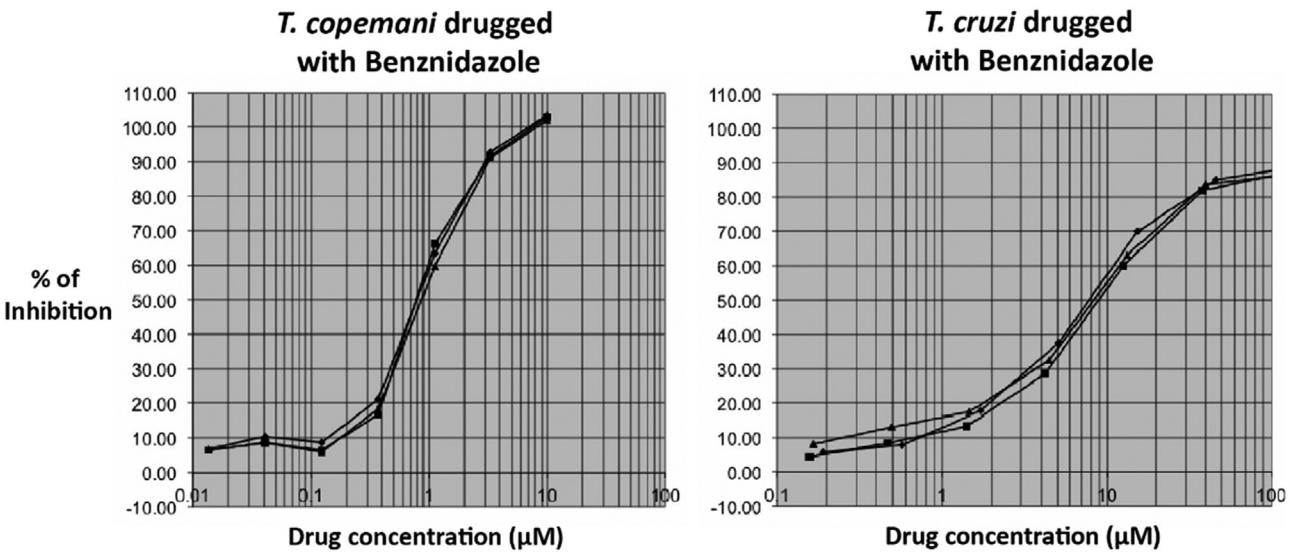


Fig. 2. Sigmoidal dose-response curves of *T. copemani* G2 and *T. cruzi* drugged with benznidazole. X-axis: percentage of inhibition. Y-axis: drug concentration.

were the most active drugs against all parasites tested. However, melarsoprol was much more active with IC50s in the sub-µM range. Significant differences in drug susceptibility between *T. copemani* G1 and G2 ($p < 0.0001$) were found. *T. copemani* G2 was more susceptible to benznidazole and melarsoprol. In contrast, *T. copemani* G1 was more susceptible to melarsoprol and miltefosine (Table 1, Fig. 3).

3.2. In vitro compound efficacy of fenarimol and pyridine derivatives

All lead compounds exhibited potent *in vitro* activity against all trypanosomes in the low and sub-µM range. However, they were more active against both strains of *T. copemani*, with the exception of PDB1 which was less active against *T. copemani* G2 than *T. cruzi* (Table 2). All four compounds exhibited similar activity against *T. cruzi*, with IC50 values ranging from 4.5 µM to 6.1 µM FN2 was the compound that presented the highest activity against both *T. copemani* G1 and G2, with IC50 of 1.122 µM for G1 and 0.969 µM for G2. There was a significant difference in susceptibility between the two *T. copemani* strains to all compounds ($p < 0.0001$), with *T. copemani* G2 more susceptible to FN1 and FN2 and *T. copemani* G1 more susceptible to FN2 and PDB2 (Table 2, Fig. 4).

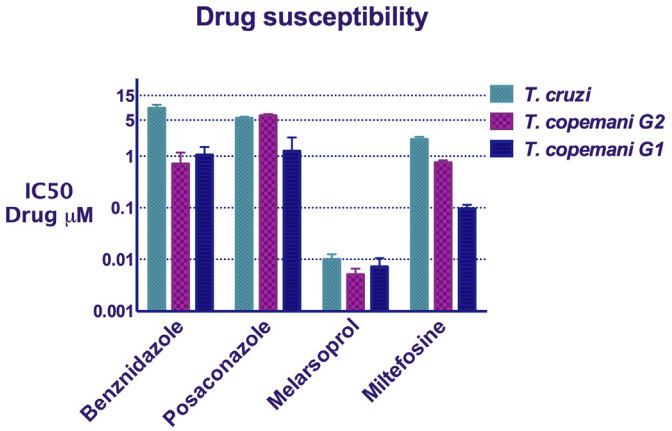


Fig. 3. Drug susceptibility of *T. copemani* G1 and G2, and *T. cruzi* against reference drugs. X-axis: IC50. Y-axis: drugs. Bars: standard deviation.

3.3. In vitro drug toxicity in L6 cells

The therapeutic index (TI) of all compounds was calculated for each parasite (Tables 1 and 2). The highest cytotoxicity for *L6* cells was exerted by melarsoprol (IC50, 0.062 µM) and miltefosine (IC50, 0.231 µM), which interestingly, had the highest activity

Table 1
Inhibitory concentration 50 (IC50) of all reference drugs against *T. copemani* G1 and G2, and *T. cruzi*, and toxicity against *L6* cells. Values are in µM. SD: standard deviation.

Compounds	*T. copemani* G1 (IC50 ± SD)	*T. copemani* G2 (IC50 ± SD)	*T. cruzi* (IC50 ± SD)	Toxicity on *L6* cells (IC50 ± SD)
Benznidazole	1.053 ± 0.183 (>94.9)	0.713 ± 0.186 (>140.2)	8.537 ± 0.306 (>11.7)	>100 µM
Posaconazole	1.254 ± 0.418 (>79.7)	6.147 ± 0.154 (>12.3)	5.429 ± 0.151 (>18.4)	>100 µM
Melarsoprol	0.007 ± 0.001 (8.8)	0.005 ± 0.0006 (12.1)	0.010 ± 0.001 (6.2)	0.062 µM
Miltefosine	0.095 ± 0.007 (2.4)	0.745 ± 0.034 (0.31)	2.109 ± 0.112 (0.1)	0.231 µM
Podophyllotoxin[a]	–	–	–	0.01 µM

() Therapeutic indices are given in parenthesis.
[a] Reference drug for toxicity.

Table 2
Inhibitory concentration 50 (IC50) of lead compounds against *T. copemani* G1 and G2, and *T. cruzi*, and toxicity against *L6* cells. Values are in μM. SD: standard deviation.

Compounds	*T. copemani* G1 (IC50 ± SD)	*T. copemani* G2 (IC50 ± SD)	*T. cruzi* (IC50 ± SD)	Toxicity on *L6* cells (IC50 ± SD)
FN1	3.316 ± 0.1021 (>30.1)	2.395 ± 0.302 (>41.7)	6.112 ± 0.0655 (>16.4)	>100 μM
FN2	1.122 ± 0.3971 (53.1)	0.969 ± 0.188 (61.4)	5.979 ± 0.2281 (10)	59.52 μM
PDB1	2.675 ± 0.7263 (>37.4)	7.178 ± 0.713 (>14)	5.261 ± 0.6828 (>19)	>100 μM
PDB2	1.51 ± 0.2736 (33.1)	3.343 ± 0.197 (15)	4.533 ± 0.3151 (11)	50.06 μM
Podophyllotixin[a]	–	–	–	0.01 μM

() Therapeutic indices are given in parenthesis.
[a] Reference drug for toxicity.

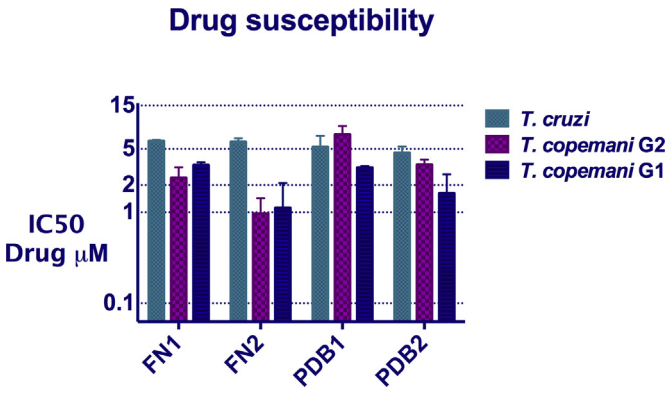


Fig. 4. Drug susceptibility of *T. copemani* G1, G2, and *T. cruzi* against lead compounds. X-axis: IC50. Y-axis: drugs. Bars: standard deviation.

against all trypanosomes as well (Table 1). Furthermore, the TI for both drugs was in general significantly low (Melarsoprol TI < 12.1 and Miltefosine TI < 2.4) suggesting the effect of the drugs was in part due to cytotoxicity instead of only to anti-trypanosomal activity (Table 1). FN2 and PDB2 compounds exhibited IC50s of 59.52 μM and 50.06 μM respectively in *L6* cells, followed by benznidazole, posaconazole, FN1 and PDB1, which exhibited IC50s higher than 100 μM. However, benznidazole and FN1 presented a better TI against both *T. copemani* strains, and PDB1 exhibited a better TI against *T. cruzi* than benznidazole and posaconazole (Tables 1 and 2).

4. Discussion

The effect of different drugs and new compounds on the growth of two strains of *T. copemani* and one strain of *T. cruzi* was investigated and compared using the AlamarBlue® assay. The AlamarBlue® assay is a sensitive and reproducible method to measure the viability of different cell lines (Ansar Ahmed et al., 1994). It has been extensively used to determine the *in vitro* activity/toxicity of different drugs against different trypanosomatids such as *T. cruzi, T. brucei* and *Leishmania* spp. (Rolón et al., 2006; Sykes and Avery, 2009; Morais-Teixeira et al., 2011; Bowling et al., 2012; Sales Junior et al., 2014; Engel et al., 2015). A previous study found AlamarBlue® was a good method to quantify the activity of different compounds against *T. brucei gambiense* and *T. b. rhodesiense in vitro* and demonstrated that results were comparable to those obtained with other fluorochrome dyes (Räz et al., 1997). Furthermore, AlamarBlue® has been shown to be slightly superior in sensitivity to the MTT cell proliferation assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), which has been extensively used in high throughput screenings (Hamid et al., 2004; Ho et al., 2012). The results of the present study showed IC50 values for benznidazole and miltefosine similar to IC50 values reported in previous studies for *T. cruzi,* confirming the reliability and reproducibility of this assay (Santa-Rita et al., 2000; Lira et al., 2001; Saraiva et al., 2002; Luna et al., 2009; Moraes et al., 2014).

The present study is the first to be carried out looking at the *in vitro* susceptibility of Australian trypanosomes to different drugs and new compounds developed against different trypanosomatids. All reference drugs, benznidazole, posaconazole, melarsoprol and miltefosine, displayed promising trypanocidal activity against the epimastigotes of both strains of *T. copemani* isolated from the critically endangered woylie and against *T. cruzi*, showing a broad anti-trypanosomal spectrum. Previous studies have also demonstrated that some of these reference drugs present a broad-spectrum of activity. Miltefosine for example, originally developed as an anticancer agent and now used for treatment of both visceral and cutaneous leishmaniasis, has also been shown to be active *in vitro* against *T. cruzi*, with an IC50 ranging from 1 μM to 3.5 μM (Santa-Rita et al., 2000; Lira et al., 2001; Saraiva et al., 2002). Melarsoprol, mainly used against late-stage sleeping sickness (Schweingruber, 2004), has also been shown to be active *in vitro* and *in vivo* against *T. lewisi* (Howie et al., 2006; Verma et al., 2011; Dethoua et al., 2013).

Miltefosine was active against *T. cruzi* and *T. copemani* G1 and G2, with IC50s of 0.095 μM, 0.745 μM and 2.1 μM respectively. However, it has been shown to present significantly lower activity *in vitro* and *in vivo* against *T. brucei* subspecies with 18-fold and 43-fold greater IC50 values of 35.5 μM for *T. brucei* and 47.0 μM for *T. brucei rhodesiense* in *in vitro* experiments (Croft et al., 1996), and 76 μM for *T. brucei gambiense* and 88 μM for *T. brucei rhodesiense* in experimentally infected mice (Konstantinov et al., 1997). The significant differences in miltefosine activity between species is not surprising if we take into account the fact that antiparasitic drugs are usually developed to target and/or inhibit intracellular signaling pathways that are crucial in cell replication and survival, and those pathways may differ between species. Hence, the significant similarities in the activity of miltefosine against both *T. cruzi* and *T. copemani* may be due to intrinsic similarities between them in the target site of the drug. However, the mechanism of action of miltefosine is not known.

Benznidazole and posaconazole demonstrated lower activity against *T. cruzi* than the drugs miltefosine and melarsoprol. Similar studies evaluating the susceptibility of different strains of *T. cruzi* to miltefosine found this drug had a greater activity against each strain than the reference drug benznidazole with IC50s ranging between 0.9 μM and 3.0 μM for miltefosine and 9.0 μM–27 μM for benznidazole (Saraiva et al., 2002; Luna et al., 2009). However, it cannot be ignored that miltefosine and melarsoprol exhibited the

highest toxicity to the mammalian cell line used and the lowest therapeutic indices. This suggests that the greater activity of both drugs against *T. cruzi* and *T. copemani* may not be entirely due to their trypanocidal activity. This is not the first study showing toxicity of melarsoprol and miltefosine in mammalian cell lines. Melarsoprol has been shown to induce programmed cell death or apoptosis in leukemic and plasma cell lines *in vitro* (König et al., 1997; Rousselot et al., 1999) as well as miltefosine in numerous tumour cell lines (Engelmann et al., 1995; Henke et al., 1998; Rybczynska et al., 2001).

All fenarimol and piridyne derivatives exhibited potent activity against *T. cruzi* and *T. copemani* epimastigotes. Moreover, these four compounds showed better activity against *T. cruzi* than the *T. cruzi* reference drug, benznidazole. These results are consistent with those obtained by Keenan (Keenan et al., 2013c), who showed that both FN1 and FN2 exhibited curative activity in mice infected with the Tulahuen strain of *T. cruzi* and significant activity *in vitro* against *T. cruzi* amastigotes, as well as low toxicity in *L6* cells. However, the *T. cruzi* IC50s of both fenarimol compounds obtained in the present study are generally higher than those previously reported (Keenan et al., 2013c). Moreover, Moraes (Moraes et al., 2014) found that amastigotes of the *T. cruzi* strains Y, CL, and Tulahuen, and the clones Dm28c, ARMA13 cl1, 92-80 cl2, and ERA cl2, exhibited better IC50s for posaconazole, PDB1 and PDB2 compared with our findings.

These discrepancies may be due to the use of different *T. cruzi* strains but most probably because different trypanosome life cycle stages were used in both studies. Several studies have revealed that some drugs or compounds, including benznidazole, are more active against *T. cruzi* intracellular amastigotes compared to epimastigotes and trypomastigotes (Freire-de-Lima et al., 2008; Luna et al., 2009; Sales Junior et al., 2014). This has also been shown with different species of *Leishmania*, where intracellular amastigotes showed greater susceptibility to miltefosine, than promastigotes (Obonaga et al., 2014). This diverse degree of activity that some drugs present in different life-stages of the parasite, has been shown to be related to the capacity of the drug to exert anti-trypanosomatid action independently of cell-mediated parasiticidal mechanisms (Vermeersch et al., 2009). For example, a greater susceptibility of *Leishmania* amastigotes than promastigotes to miltefosine was suggested to be the result of increased cytotoxicity within the macrophage, conferred by alkyl-lysophospholipids promoting the death of intracellular parasites as a secondary effect on host cells by oxidative burst or production of reactive-oxygen metabolites (Azzouz et al., 2005). Although, we previously demonstrated that *T. copemani* G2 is able to invade *L6* and *VERO* cells *in vitro*, the parasite was not able to replicate inside cells (Botero et al., 2016). Therefore, testing the drugs on intracellular amastigotes could not be achieved. The use of a better *in vitro* model, possibly a marsupial derived cell line, that could support the intracellular growth of *T. copemani* will be necessary to test all drugs on amastigotes. Moreover, complementary *in vivo* studies using a murine or any other *in vivo* model are required as a next step to better understand *T. copemani* drug susceptibility on natural hosts.

T. copemani G1 and G2, although grouped within the same clade in a phylogeny, exhibited genetic differences in both the 18SrDNA and g*GAPDH* genes (Botero et al., 2013). Furthermore, *T. copemani* G1 has always been found in blood of marsupials while *T. copemani* G2 has also been found in tissues (Botero et al., 2013). Interestingly, we found that both strains of *T. copemani* exhibited significant differences in susceptibility to the different drugs used, supporting previous findings that genetic variation/gene varinats within species determines the degree of susceptibility to drugs (Campos et al., 2011; Plourde et al., 2012; Graf et al., 2013, 2016; Laffitte et al., 2016). Previous studies have shown an association between *T. cruzi* genetic diversity and their susceptibility to different drugs. Moraes (Moraes et al., 2014) reported a different response to the drugs benznidazole, posaconazole, EPL-BS967 (PDB1), and EPL-BS1246 (PDB2) among genetically different *T. cruzi* strains from all different DTUs (discrete typing units). Moreover, the observation of differences in susceptibility to benznidazole among several *T. cruzi* strains isolated from humans, vectors and marsupials has also been reported (Toledo et al., 1997). Phylogenetic studies have shown considerable intra-specific genetic variability within *T. copemani* isolates and the presence of co-infections with different *T. copemani* genotypes/strains in naturally infected animals (Botero et al., 2013). This variability and its possible association with the different phenotypic responses to drugs may complicate the scenario and may have important consequences on future attempts to combat *T. copemani* infections.

Finally, the fact that benznidazole and FN2 had better therapeutic indices against *T. copemani* G1 and G2 compared with the other drugs (Benznidazole TI > 94.9 and TI > 140.2; FN2 TI > 53.1 and TI > 61.4) suggests these drugs could be potentially used as possible therapeutics for ameliorating the clinical effects of infections with this parasite in wildlife. However, *in vivo* trials are needed before they can be used in wildlife.

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