Differential in vitro activity of the DNA topoisomerase inhibitor idarubicin against *Trypanosoma rangeli* and *Trypanosoma cruzi*

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In this study the effect of eight DNA topoisomerase inhibitors on the growth Trypanosoma rangeli *epimastigotes in cell culture was investigated. Among the eight compounds tested, idarubicin was the only compound that displayed promising trypanocidal activity with a half-maximal growth inhibition (GI_{50}) value in the sub-micromolar range. Fluorescence-activated cell sorting analysis showed a reduction in DNA content in* T. rangeli *epimastigotes when treated with idarubicin. In contrast to* T. rangeli*, against* Trypanosoma cruzi *epimastigotes idarubicin was much less effective exhibiting a GI_{50} value in the mid-micromolar range. This result indicates that idarubicin displays differential toxic effects in* T. rangeli *and* T. cruzi*. Compared with African trypanosomes, it seems that American trypanosomes are generally less susceptible to DNA topoisomerase inhibitors.*

Key words: *Trypanosoma rangeli* - *Trypanosoma cruzi* - Chagas disease - DNA topoisomerase inhibitors - drug screening - chemotherapy

Chagas disease is caused by the protozoan parasite *Trypanosoma cruzi* and occurs mainly in Central and South America. Approximately 10 million people are infected with the parasite and in 2008 the disease killed more than 10,000 individuals (WHO 2010). Only two drugs, benznidazole and nifurtimox, are available for treatment of Chagas disease (Urbina & Docampo 2003, WHO 2010). Both drugs were developed 40 years ago and are only effective in the acute phase of the disease (WHO 2010). In addition, both remedies have significant side effects, ranging from nausea to life-threatening complications (Urbina & Docampo 2003). Thus, the development of new drugs for treatment of Chagas disease is urgently required.

One strategy to identify new chemotherapies for treatment of Chagas disease is the screening of existing drugs for antichagasic activity. In this context, DNA topoisomerase and proteasome inhibitors approved for cancer chemotherapy have been shown to display promising trypanocidal activities (Deterding et al. 2005, Steverding & Wang 2009). Moreover, previous studies have shown that bacterial topoisomerase inhibitors block proliferation and differentiation of *T. cruzi* (Pate et al. 1986, Gonzales-Perdomo et al. 1990). The aim of this study was to investigate whether commercially available eukaryotic DNA topoisomerase inhibitors show anti-trypanosomal activities against American trypanosomes.

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DNA topoisomerases are essential enzymes that catalyse topological changes in DNA and therefore play key roles in replication, transcription, recombination and chromosome condensation (Corbett et al. 2004, Bates & Maxwell 2005). Two types of topoisomerase have been characterised: type I topoisomerases introduce transient single-strand breaks in DNA, whereas type II topoisomerases produce transient double-strand breaks (Berger et al. 1996, Stewart et al. 1998). Topoisomerases are critical to completion of successful cell cycles and, therefore, have been developed as drug targets both for antimicrobial and anticancer chemotherapy. Most anticancer topoisomerase inhibitors (anthracyclins, camptothecins, mitoxantrone and etoposide) poison topoisomerases by inhibiting the DNA religation activity of the enzymes (Pommier et al. 2010). In addition, if anti-cancer drugs targeting topoisomerases prove effective in killing *T. cruzi*, a more rapid application for treatment of Chagas disease with less extensive clinical trials might be possible as their in vivo toxicities are already well established.

Trypanosoma rangeli is a New World trypanosome species which is non-pathogenic for mammals and is frequently found to be infecting humans (Guhl & Vallejo 2003). Its geographical distribution overlaps with that of *T. cruzi* and it shares the same vertebrate hosts and insect vectors. *T. rangeli* is closely related to *T. cruzi* with similar morphology and antigenicity which can complicate diagnosis. Phylogenetic analyses indicate that although each of these sibling species have discrete monophyletic origins they share a common origin and group closely together to the exclusion of other trypanosomes (Stevens et al. 1999, Ortiz et al. 2009). Moreover, both species show considerable genetic heterogeneity. *T. cruzi* diversity is currently encompassed in six disease typing units (DTUs) I-VI (Zingales et al. 2009) where DTU I (TcI) and DTU II (TcII) are most divergent from one another

(Westenberger et al. 2005). In addition, TcI is the most abundant and widely dispersed of all the *T. cruzi* DTUs in the Americas while TcII is predominantly found in southern and central regions of South America (Zingales et al. 2012). Moreover, TcII associated with megasyndromes, as well as cardiac manifestations, has been isolated mainly from domestic transmission (Zingales et al. 2012). For these reasons, DNA topoisomerase inhibitors were initially screened with *T. rangeli* and effective compounds were then tested for their activity against two *T. cruzi* strains, one from DTU I (Sylvio X10) and the other from DTU II (Esmeraldo).

The trypanocidal activity of eight DNA topoisomerase inhibitors used as anticancer drugs was evaluated in a growth assay with epimastigotes of *T. rangeli* (Choachi strain) (Grisard et al. 1999). In brief, cells were seeded in 24-well plates in a final volume of 1 mL liver infusion tryptose medium plus 15% heat-inactivated foetal calf serum (Grisard et al. 1999) containing various concentrations of DNA topoisomerase inhibitors (10^{-4}-10^{-9} M) dissolved in 100% dimethyl sulfoxide (DMSO). The controls contained DMSO alone. In all experiments, the final DMSO concentration was 1%. The seeding densities were 0.6-1 × 10^6 parasites per mL. After 24 h incubation at 27ºC, live cells were counted using a haemocytometer. The 50% growth inhibition value (GI_{50}), i.e. the inhibitor concentration necessary to reduce the growth rate of the cells to half of that of controls was determined by linear interpolation using the following equation (Huber & Koella 1993):

$$\log(GI_{50}) = \log(x_1) + \frac{(y_1 - y_0/2)}{(y_1 - y_2)} \times [\log(x_2) - \log(x_1)]$$

where x_1 is the drug concentration at where the cell density y_1 is more than half of the density y_0 found in the control and x_2 is the drug concentration at where the cell density y_2 is less than half of the control. The minimum inhibitory concentration (MIC), i.e. the lowest concentration of the inhibitor at which all cells were killed, was determined microscopically.

With the exception of the anthracyclines aclarubicin and idarubicin, all other DNA topoisomerase inhibitors displayed no activity against *T. rangeli* epimastigotes (Table I). Only idarubicin exhibited promising trypanocidal activity (Fig. 1) with GI_{50} values in the sub-micromolar range (Table I). Compared with ketoconazole, a well-known antifungal and antiparasitic agent, idarubicin was 50 times more effective against *T. rangeli* (Table I). That most of the DNA topoisomerase inhibitors exhibited little or no activity was unexpected as this class of compounds was previously shown to be very effective against *Trypanosoma brucei* bloodstream forms with GI_{50} values ranging from 3-20 µM (Deterding et al. 2005). In addition, the anthracenedione mitoxantrone was recently reported to induce an inhibitory effect on cellular proliferation of *T. cruzi* epimastigotes with a GI_{50} value in the low micromolar range (Zuma et al. 2011). That the two camptothecin analogues, topotecan and irinotecan, showed no activity against *T. rangeli*, may be due to the fact that both inhibitors are hydrophilic compounds (Rothenberg 1997). However, to prove this hypothesis, additional experiments are needed to be performed. Likewise, both drugs showed only weak activity against *T. brucei* bloodstream forms (Deterding et al. 2005). However, the parent compound of topotecan and irinotecan, camptothecin, was reported to significantly inhibit the growth of *T. brucei* bloodstream forms and *T. cruzi* epimastigotes with GI_{50} values of around 0.4 and 2.1 µM, respectively (Bodley & Shapiro 1995, Deterding et al. 2005, Zuma et al. 2011).

As G2/M arrest is a well-documented effect of topoisomerase II inhibitors (Larsen et al. 2003) we studied the impact of idarubicin on cell cycle distribution in *T. rangeli*. Epimastigote forms of *T. rangeli* were incubated

TABLE I

Minimum inhibitory concentration (MIC) and growth inhibition values (GI_{50}) of DNA topoisomerase inhibitors for *Trypanosoma rangeli*

Compound	Target	n	MIC (µM)	GI_{50} (µM)
Anthracyclines				
Aclarubicin	Topo I, II, proteasome	3	100	20.9 ± 8.8
Doxorubicin	Topo II	2	> 100	> 100
Epirubicin	Topo II	2	> 100	> 100
Idarubicin	Topo II	3	100	0.49 ± 0.27
Camptothecins				
Irinotecan	Topo I	3	> 100	> 100
Topotecan	Topo I	3	> 100	> 100
Miscellaneous				
Etoposide	Topo II	3	> 100	> 100
Mitoxantrone	Topo II	2	> 100	> 100
Antichagasic drug				
Ketoconazole	Ergosterol	7	> 100	25.7 ± 12.9

with DMSO (control) or 10 µM idarubicin, a concentration 10-fold lower than the MIC value (Table I). After 18 h incubation, the cells were washed with PBS/1% glucose and fixed in ice-cold methanol (Ormerod 2000). Then, cells were stained with 50 µg/mL propidium iodide in water and analysed with a BD Accuri C6 flow cytometer. Idarubicin failed to arrest *T. rangeli* in G2/M as is evident from the disappearance of the G2/M cell population (Fig. 2). Instead, idarubicin treatment resulted in a reduction of DNA content in many cells (Fig. 1) (sub G1 cell population). A similar result was also obtained with 1 µM idarubicin although DNA reduction was not so pronounced as with 10 µM idarubicin (data not shown). No difference in cell cycle distribution compared to control cells was seen with 0.1 µM idarubicin (data not shown). This is reminiscent of the observation for doxazolidine-treated mammalian cells where apoptosis is induced and DNA is degraded (Kalet et al. 2007). These data suggest that idarubicin's mechanism of cytotoxicity is probably topoisomerase II independent.

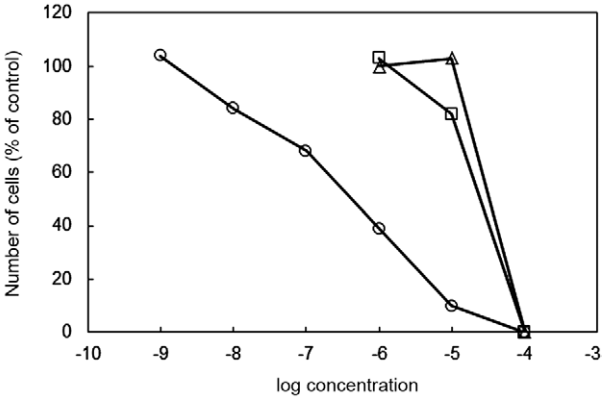

Fig. 1: effect of idarubicin on the growth of *Trypanosoma rangeli* and *Trypanosoma cruzi*. Epimastigotes of *T. rangeli* (circles) and *T. cruzi* Sylvio X10 (squares) and Esmeraldo (triangles) strains were incubated with varying concentrations of idarubicin. After 24 h of culture, motile cells were counted using a haemocytometer. Mean values of three experiments are shown.

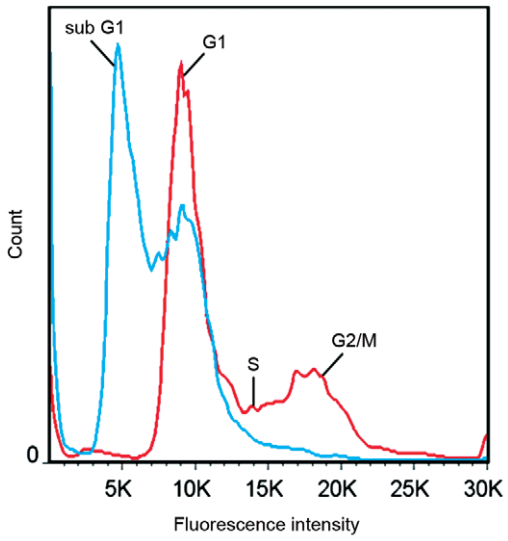

Fig. 2: cell cycle distribution of *Trypanosoma rangeli* exposed to idarubicin. Epimastigotes of *T. rangeli* were treated with 10 µM idarubicin (blue line) or vehicle (dimethyl sulfoxide, red line) and stained with propidium iodide. DNA content was analysed by flow cytometry.

Next, the effect of idarubicin on epimastigotes of two *T. cruzi* strains, Sylvio X10 and Esmeraldo, was tested using the same growth assay as described for *T. rangeli*. The seeding densities ranged between 0.9-1.3 × 10^6 parasites per mL. Both *T. cruzi* strains were less susceptible to idarubicin than *T. rangeli* (Fig. 1) with GI_{50} values in the mid-micromolar range (Table II). Based on the GI_{50} values, the Sylvio X10 strain was slightly more resistant (1.5 times) towards idarubicin than the Esmeraldo strain ($p = 0.052$).

Our results when compared with those published for the African trypanosomes (Deterding et al. 2005) indicate that American trypanosomes are less sensitive to DNA topoisomerases inhibitors. That DNA topoisomerase inhibitors affect *T. rangeli* and *T. cruzi* differently to *T. brucei* has important implication for the potential use of this class of drugs as broad-spectrum trypanocides. The differences in susceptibility towards DNA topoisomerase inhibitors between American and African trypanosomes may have a variety of causes. First, there may be a difference in the uptake of the drugs by the different trypanosome species. All DNA topoisomerase inhibitor tested in this study are lipophilic compounds and, therefore, should be able to enter cells by passive diffusion. As the diffusion rate is a function of temperature, the bloodstream forms of *T. brucei* cultivated at 37ºC could be expected to take up the drugs more quickly than epimastigotes of *T. rangeli* and *T. cruzi* grown at 27ºC. Second, the different life-cycle stages of trypanosomes (mammalian vs. insect) may have different sensitivities towards DNA topoisomerase inhibitors. Third, inhibition of topoisomerases is predicted to affect bloodstream forms of *T. brucei* to a greater extent as they have a faster proliferation rate compared to epimastigotes of *T. rangeli* and *T. cruzi*.

Since the molecular inhibition mechanism of idarubicin is not different from that of the other anthracyclines tested in this study (Plumbridge & Brown 1978), why is idarubicin the only compound displaying trypanocidal activity against *T. rangeli*? The answer to this question may

TABLE II

Minimum inhibitory concentration (MIC) and growth inhibition values (GI_{50}) of idarubicin for *Trypanosoma cruzi*

Strain	n	MIC (µM)	GI_{50} (µM)
Sylvio X10 (DTU I)	3	100	38.5 ± 6.9
Esmeraldo (DTU II)	3	100	24.6 ± 2.4

DTU: disease typing unit.

lie in the structure of the molecules. Idarubicin differs from doxorubicin and epirubicin by the deletion of a methoxy group at the position C-4 of the basic anthracycline ring scaffold. This modification results in a higher lipophilic coefficient with the effect that idarubicin is taken up more rapidly and induces more DNA single strand breaks (Supino et al. 1977, Schwartz & Kanter 1981). The trypanocidal activity of aclarubicin (which has a hydroxyl group at position C-4 and therefore should be inactive) can be explained by the fact that it also inhibits DNA topoisomerase I (Bridewell et al. 1997) and the proteasome (Figueiredo-Pereira et al. 1996). Mitoxantrone has hydroxyl groups at position C-1 and C-4 of the anthracenedione ring scaffold which would make it less lipophilic explaining its inactivity. These structure-activity relationships suggest that in order to exhibit trypanocidal activity DNA topoisomerase inhibitors should be highly lipophilic.

Although *T. rangeli* and *T. cruzi* are considered sibling species, for some drugs such as nifurimox it appears that they have similar susceptibility (Marinkelle 1982). However, this is clearly not always the case (Avila et al. 1981). Here we have demonstrated a significant difference in drug susceptibility to idarubicin, a drug which displayed substantial trypanocidal activity against *T. rangeli*, but not against *T. cruzi*. Bioinformatics analysis does suggest some significant differences in the topoisomerase repertoire between *T. rangeli* and *T. cruzi* (EC Grisard, unpublished observations) and this heterogeneity may well be the reason for the difference in susceptibility that we observe. These findings reinforce the view that although the use of *T. rangeli* as a "laboratory safe" surrogate for *T. cruzi* in drug screening and pre-screening is appealing and may well be useful, where it is used the results should be interpreted with care. In addition, our results also indicate that the use of insect forms has drawbacks for screening potential drugs for Chagas disease because these life cycle stages can have different sensitivities than mammalian forms to antichagasic agents.

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