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Trypanocidal activity of genotoxic concentration of benznidazole on epimastigote forms of *Trypanosoma cruzi*

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ABSTRACT. The genotoxicity of benznidazole at a concentration of 75 μM, used in the treatment of Chagas' disease, has been recently reported. The present study evaluated the inhibitory effect of benznidazole on the growth of epimastigote forms of *T. cruzi* I and II by using genotoxic (75 μM) and non-genotoxic (50 μM) concentrations. To assess the growth rates of *T. cruzi* strains G2, A2.1A, CL, Y, and 2052, parasites in the epimastigote form were cultured in LIT medium for 192 h at 28ºC, with (50 and 75 μM) and without (negative control) benznidazole. Benznidazole at both concentrations inhibited all the strains, regardless of genetic group. In the 75 μM concentration, there was a significant decrease in the number of parasites inoculated at T_0 after 96 h incubation. The results showed that although genotoxic and non-genotoxic doses of benznidazole inhibit the growth of the epimastigote forms of *T. cruzi* I and II, only the 75 μM dose seem to indicate a possible trypanocidal effect.

Keywords: benznidazole, *T. cruzi* I, *T. cruzi* II, Chagas' disease, genotoxicity.

Atividade tripanocida da concentração genotóxica do benzonidazol em formas epimastigotas de *Trypanosoma cruzi*

RESUMO. O benzonidazol é um medicamento utilizado no tratamento da doença de Chagas, cuja genotoxicidade foi recentemente observada em concentrações a partir de 75 μM. O efeito inibitório do benzonidazol sobre o crescimento de formas epimastigotas de *T. cruzi* I e II foi avaliado no presente trabalho, utilizando-se concentrações genotóxica (75 μM) e não genotóxica (50 μM) deste medicamento. Para avaliação da taxa de crescimento das cepas G2, A2.1A, CL, Y e 2052, os parasitos na forma epimastigota foram cultivados em meio LIT, durante 192 horas, à 28 º C, tanto em presença de benzonidazol (50 e 75 μM), quanto em sua ausência (controle negativo). O efeito inibitório do benzonidazol, em ambas concentrações, foi observado para todas as cepas analisadas, independentemente do grupo genético a que pertençam. Na concentração de 75 μM, observou-se após 96 horas de incubação, redução significativa do número de parasitos inoculados no tempo zero (T_0). Os resultados demonstraram que tanto a dose genotóxica quanto a não genotóxica do benzonidazol inibiram o crescimento de formas epimastigotas de *T. cruzi* I e II, porém somente a dose de 75 μM pode indicar um possível efeito tripanocida.

Palavras-chave: benzonidazol, *T.cruzi* I, *T.cruzi* II, doença de Chagas, genotoxicidade.

Introduction

Although the Brazilian Health Ministry has recently received international certification of the interruption of the transmission of Chagas' disease by the vector *Triatoma infestans* in all the states of Brazil (BRASIL, 2006), medical assistance to already infected people is still important. Current data suggest that approximately 7.5 million people are infected with *Trypanosoma cruzi* (OPS, 2006). Chagas' disease can be chronic, with weakening effects on the patient. Several investigators insist that the presence of the parasite in the patient is an important factor in the maintenance and clinical evolution of the disease, which underpins all etiological treatment (HIGUCHI et al., 1993; BRENER, 2000; SUASNABER et al., 2000; COURA; CASTRO, 2002; GARCIA et al., 2005). Benznidazole is the only treatment available in Brazil which affects all the evolutionary forms of *Trypanosoma cruzi* (COURA; CASTRO, 2002; PINTO DIAS, 2006).

The medical practitioner must follow up the etiological treatment, owing to the possible occurrence of digestive changes, dermatitis, neuritis, and leucopenia (CANÇADO, 1997; FRAGATA FILHO et al., 1997; ABAD-FRANCH et al., 2010). The incidence of such side effects is variable depending on the age of the patient (less frequent in younger patients), geographic regions and the quality of

the clinical supervision of the treatment (RASSI JUNIOR et al., 2009).

In addition to these side effects, benznidazole may produce lymphomas in mice (TEIXEIRA et al., 1994), and other types of neoplasic diseases in immunosuppressed heart-transplant patients (BOCCHI et al., 1998). The production of lymphomas in the murine model is rather controversial. In their experiments with mice, Teixeira et al. (1994) noted a high incidence of lymphomas in mice treated with benznidazole; whereas Andrade et al. (2003) failed to observe any lymphomas or other types of cancer.

Kaneshima and Castro-Prado (2005) evaluated the carcinogenic potential of benznidazole by inducing somatic crossing-over in heterozygous diploid cells of *Aspergillus nidulans*. The process caused homozygosis of recessive or deleterious genes and reduced the constitutional heterozygosity of tumor-suppressor genes, giving rise to neoplasia (LASKO et al., 1991; ZIMMERMANN, 1992; BEUMER et al., 1998). Of the three benznidazole concentrations evaluated, concentrations of 100 μM and 75 μM were found to be genotoxic, and the concentration of 50 μM non-genotoxic. These investigators showed that the genotoxicity of benznidazole is dose-dependent for the induction of mitotic crossing-over (KANESHIMA; CASTRO-PRADO, 2005).

The existence of two major genetic lineages of *T. cruzi*, denominated *T. cruzi* I and *T. cruzi* II, is based on different methodologies and is well established (ANONYMOUS, 1999; STURM; CAMPBELL, 2010). The *T. cruzi* II lineage has five genetic subdivisions: *T. cruzi* IIa, b, c, d, e (BRISSE et al., 2001; STURM; CAMPBELL, 2010). These lineages differ with regard to virulence in mice, infectivity in cell culture, transmissibility by triatomines, and *in vitro* and *in vivo* susceptibility to drugs (LAURENT et al., 1997; LANA et al., 1998; REVOLLO et al., 1998; TOLEDO et al., 2003; MORTARA et al., 2005; STURM; CAMPBELL, 2010).

The present study evaluated the inhibitory effect of benznidazole on the growth of epimastigote forms of *T. cruzi* I and II, by employing genotoxic and non-genotoxic doses of the anti-parasite agent.

Material and methods

Parasites

Table 1 lists the *T. cruzi* strains used in this study, their hosts and genetic lineages, and the *in vivo* susceptibility to benznidazole of three of them as described by Filardi and Brener (1987); Toledo et al. (1997).

Table 1. *In vivo* susceptibility to benznidazole of strains of *Trypanosoma cruzi* isolated from different hosts and belonging to different genetic lineages.

Strain	Host	Genetic lineage	*In vivo* susceptibility to benznidazole
G2	*Didelphis* sp	*T. cruzi* I	ND
A2.1A	*Triatoma sordida*	*T. cruzi* I	resistant
CL	*Triatoma infestans*	*T. cruzi* IIe	totally sensitive
Y	human	*T. cruzi* IId	partially sensitive
2052	human	*T. cruzi* II	ND

ND = not done.

Inhibitory effect of benznidazole on growth of *T. cruzi* I and II epimastigote forms

Benznidazole was dissolved in Liver Infusion Tryptose (LIT) medium and supplemented with 10% bovine fetal serum to concentrations of 50 μM and 75 μM, non-genotoxic and genotoxic respectively, following Kaneshima; Castro-Prado (2005). Experiments were carried out in triplicate. Approximately 1 x 10^6 parasites mL^{-1} of each strain, in an exponential growth phase, were seeded in tubes with 3 mL of LIT without benznidazole (negative control) and with 50 and 75 μM benznidazole (test). Cultures were kept at 28ºC and parasite growth was evaluated by counting in a Neubauer chamber using a 5% formaldehyde solution. Counting was undertaken between 0 and 192 h of incubation, at 24-h intervals. The inhibitory effect was estimated by the difference of parasite growth at each period, in the absence and in the presence of the drug, and expressed in parasites/mL of the culture. Growth rates of epimastigotes with 50 and 75 μM benznidazole or without benznidazole were compared by Student's t-test, at the 5% significance level.

Results and discussion

Many authors have explored possible links between the phylogenetic diversity of *T.cruzi* and biological properties (LAURENT et al., 1997; LANA et al., 1998; TOLEDO et al., 2002; TOLEDO et al., 2003). Correlations between the susceptibility to benznidazole and genetic groups of *T.cruzi* have been described (TOLEDO et al., 2003). In the present study, five *T. cruzi* strains isolated from different host species, belonging to genetic lineages *T. cruzi* I (G2, A2.1A), *T. cruzi* II (2052), *T. cruzi* IId (Y), and *T. cruzi* IIe (CL), and showing different levels of susceptibility to benznidazole, were used.

Figures 1 and 2 show that all the strains were affected by benznidazole at the 50 and 75 μM concentrations. The growth rate of the parasites was significantly reduced ($p < 0.05$) compared to the negative control. This decrease in growth rate may be associated with the activity of benznidazole, which interferes with protein synthesis or interacts with DNA and RNA molecules

(CANÇADO, 1985; DIAZ-TORANZO et al., 1988; BRENER, 2000; MAYA et al., 2004; MAYA et al., 2007). The inhibition of the *in vitro* growth of epimastigotes in all the strains corroborates the observations of Cuéllar et al. (2003). These authors studied epimastigote forms of *T. cruzi*, and determined the IC_{50} to be approximately 10 μM, equivalent to a concentration around 3 μg mL^{-1}.

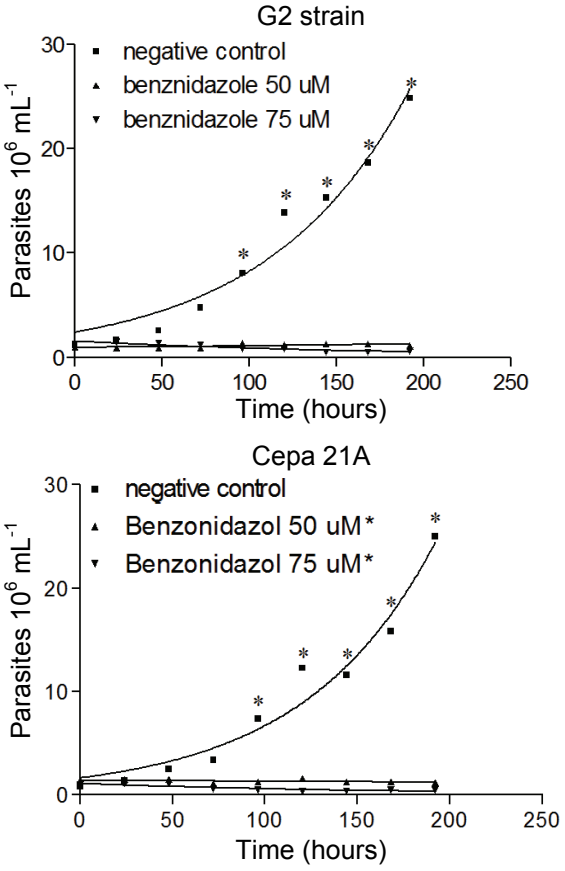

Figure 1. The inhibitory effect of benznidazole on the growth rate of epimastigotes of *Trypanosoma cruzi* I strains without (negative control) and with the drug (50 and 75 μM). *significantly different, $p < 0.05$.

When growth rates of the two lineages of *T. cruzi* were compared in pairs, without benznidazole (negative control), no significant difference was found between them. No significant difference has been reported in the inhibitory effect of benznidazole with regard to the genetic lineage to which the strain belongs or considering the degree of *in vivo* susceptibility (resistant, or partially or totally sensitive). A2.1A strain was classified as resistant to benznidazole (Table 1), although, according to figure 1, cell growth of epimastigotes of this strain was inhibited by benznidazole at concentrations 50 and 75 μM. Similar results were obtained for strains G2; CL; Y and 2052 (Figures 1 and 2). These results are in agreement with Villarreal et al. (2004) and Luna et al. (2009).

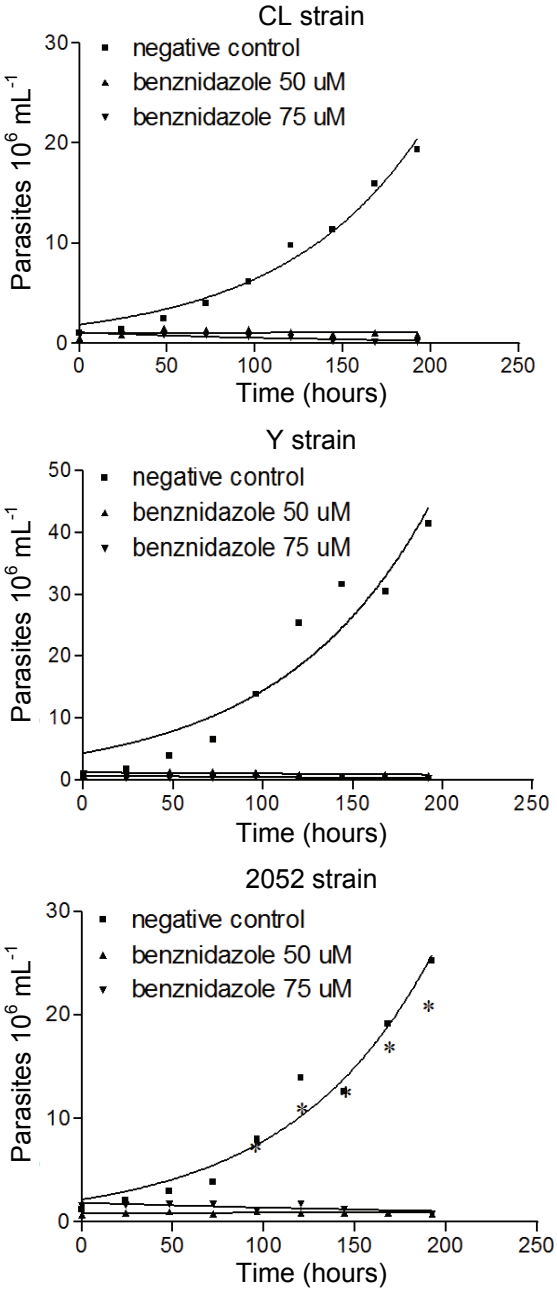

Figure 2. The inhibitory effect of benznidazole on the growth rate of epimastigotes of *Trypanosoma cruzi* II strains without (negative control) and with the drug (50 and 75 μM). * significantly different, $p < 0.05$.

Several researchers have reported associations between the biological traits of the strains and their phenotype and genotype (ANDRADE et al., 1997; REVOLLO et al., 1998; TOLEDO et al., 2002; TOLEDO et al., 2003). However, the statistical lack of association between the strains' biological characteristics and their genetic diversity in the present study agrees with the results reported elsewhere (ANDO et al., 2006; BERTOLI et al., 2006; VILLARREAL et al., 2005).

Figure 3 shows that the number of epimastigotes at different time intervals was similar to that at T_0. In other words, it was similar to the number of epimastigotes inoculated in LIT medium with benznidazole at concentration 50 μM. A significant decrease in the number of epimastigotes inoculated in LIT medium with 75 μM of benznidazole, after 96 h incubation, was verified. Similar results were obtained for strains CL and Y (sensitive and partially sensitive to benznidazole; data not shown).

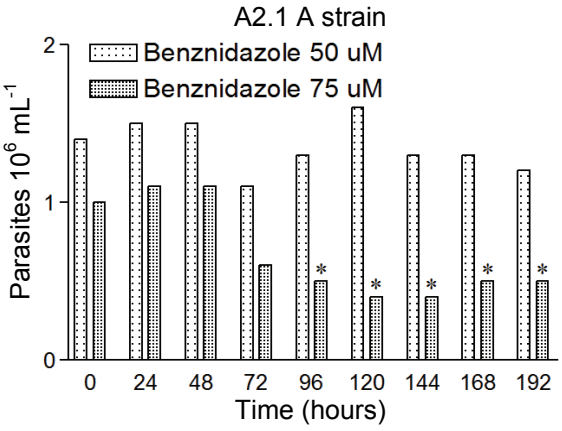

Figure 3. Cell growth rate of *Trypanosoma cruzi* I (A21A strain) in LIT medium with benznidazole concentrations of 50 and 75 μM. *significantly different from T_0, $p < 0.05$.

The decrease in epimastigotes in the 75μM concentration may be a possible trypanocidal effect of benznidazole, similar to that described by Cançado (1985) for a concentration of 100 μg mL^{-1}. Because Cançado (1985) stated that all the parasites were lethally affected, it should be emphasized that the concentration used was approximately four times higher than that of 75 μM.

Several studies evidenced that no etiological treatment is totally efficient in the chronic phase of Chagas' disease (FILARDI; BRENER, 1987; BRENER, 2000; PRATA, 2001; COURA, 2009). The presence of the parasite is important in the maintenance and clinical evolution of the disease in chronic patients, especially those with heart conditions caused by Chagas' disease. In fact, this condition is accountable for many cases of early retirement or loss of working hours due to sick leave, which causes serious economic losses (HIGUCHI et al., 1993; SUASNABER et al., 2000).

In spite of the low efficiency of benznidazole in the treatment of these patients, many investigators are in favor of etiological treatment of the disease because it is linked with improvement in health and improved prospects for the individual's survival (VIOTTI et al., 1994; ANDRADE et al., 1996; CANÇADO, 2002; ANDRADE et al., 2004; GARCIA et al., 2005; VIOTTI et al., 2006). The negative reactions and side effects of benznidazole should also be taken into account, because they may require the interruption of the treatment (CANÇADO, 1997; FRAGATA-FILHO et al., 1997).

Kaneshima and Castro-Prado (2005) registered a discrete genotoxic effect of a 75 μM concentration of benznidazole on *A. nidulans*. These investigators showed that mitotic crossing-over was observed in only one of the genetic intervals, and in only one of the benznidazole-treated diploid strains examined.

The non-genotoxic concentration (50 μM) of benznidazole is similar to plasmatic level during chemotherapy treatment in humans, as noted by Villarreal et al. (2005). Since *Trypanosoma cruzi* I (A21A strain) cell growth rate in LIT medium was inhibited by benznidazole at concentration 50 μM, and at 75 μM benznidazole dose, a significant decrease in epimastigotes occurred (Figure 3). The above results will assist at understanding the limited efficacy of benznidazole in the treatment of Chagas' disease, especially in chronic patients. As previously shown, this is due to the fact that benznidazole at 50 μM concentration inhibits the growth of the parasite. At this concentration the drug has no trypanocidal effect or any radically deleterious effects on epimastigotes forms of *T.cruzi*.

In order to improve the etiological treatment of patients with chronic Chagas' disease, complementary *in vitro* and *in vivo* studies are needed to provide further information on the effects of benznidazole.

Conclusion

Current investigation showed that concentrations of 50 and 75 μM inhibit the cell growth rate of epimastigotes forms of *T. cruzi*, and that benznidazole at concentration 75 μM demonstrated a possible trypanocidal effect.

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