ANEXO A

Benznidazole Clearance of *Trypanosoma cruzi* Human Infection does not Correlate with Parasite Drug Susceptibility: A 10-Year Prospective Study

Running title:

Benznidazole susceptibility of human *T. cruzi* strains

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ABSTRACT

The nitroheterocyclic compound benznidazole (BZ) is one of the two drugs used to treat Chagas disease, one of the most important parasitic infections in Latin America. Nevertheless, therapeutic failures of BZ were reported, which were mostly attributed to different susceptibilities of *Trypanosoma cruzi* strains to this drug. To investigate this possibility, we standardized an *in vitro* test to quantify the drug activity. For twelve *T. cruzi* strains, the 50% inhibitory concentration (IC_{50}) varied from 7 to 127 µM. In parasites sub-cultured during four months the IC_{50} value showed minimal fluctuations. The test was then applied to isolates retrieved from seven chronic patients submitted to BZ therapy. The IC_{50} of the pre-treatment isolates from three patients considered cured by several criteria varied from 19 to 35 µM. Similarly, pre-treatment isolates from four non-cured patients presented IC_{50s} of 15.6 to 51 µM. We also analyzed BZ activity in parasites obtained after chemotherapy. The isolates from one patient maintained the IC_{50} value, whereas in two patients a 2-fold decrease and in one patient a 3-fold increase in BZ susceptibility was observed. Possible selection of parasite sub-populations during or after treatment was investigated based on the profile of nine microsatellite *loci*. Our results show for the first time that the susceptibility to BZ of the infecting *T. cruzi* population is not predictive of cure and support the notion that in addition to the direct role in parasite clearance, the effect of BZ on the host immune response determines chemotherapy efficacy.

INTRODUCTION

Human Chagas disease has been affecting people from nearly all countries of Latin America. Due to the continuous entry of infected immigrants from disease endemic countries, Chagas disease now is becoming an important health concern also in USA and Europe. Throughout Latin America, the transmission of the protozoan parasite *Trypanosoma cruzi*, the causative agent of Chagas disease, has been steadily reduced through a series of multinational initiatives aiming at the elimination of domestic vectors in extensive areas and screening of blood donors for *T. cruzi* infection. As result, the global disease prevalence has been reduced from the 1990 estimates of 16–18 million people infected to approximately 10 million, according to recent data from the Disease Control Priorities Project of the NIH and World Bank (34). Nevertheless, this number most probably is underestimated because more extensive serological surveys need to be conducted in several countries of Latin America.

T. cruzi is predominantly transmitted by hematophagous insects of the Reduviidae family, but also through blood transfusion, organ transplantation, congenitally and through the ingestion of contaminated food. The parasite has a complex life cycle characterized by several developmental forms in both the mammalian host and insect vector. Three stages are classically described: the epimastigotes, residing in the vector's digestive tract; the trypomastigotes, found in the insect rectum and mammalian blood circulation; and the amastigotes, encountered in the cytoplasm of the mammalian host cell.

Chagas disease progresses through two successive stages: the acute phase with patent parasitemia and the chronic phase characterized by subpatent parasitemia and scarce tissue parasitism. The majority of chronic individuals are asymptomatic (indeterminate form). On the other hand, up to 40% of patients develop chronic chagasic cardiomyopathy or gastrointestinal disorders predominantly in the esophagus and colon. Accumulating evidence indicates that parasite persistence, in concert with an unbalanced immune response, plays a central role in the development of the pathology observed in both acute and chronic human Chagas disease. Accordingly, the prevailing opinion is that this disease should be treated with anti-parasitic compounds (reviewed in 15, 48).

Two nitroheterocyclic drugs have been used for specific treatment of *T. cruzi* infection: nifurtimox (NF) (launched by Bayer in 1967, Lampit®), and benznidazole (BZ) (launched by Roche in 1972; Rochagan® and Radanil®) (reviewed in 9). NF is a nitrofuran (3-methyl-N-[(5-nitro-2-furanyl)-methylene]-4-thiomorpholinamine-1,1-dioxide) and BZ is a nitroimidazole derivative: ([N-benzyl-2-nitro-1-imidazole acetamide). These agents function

as prodrugs and must undergo nitoreductases-mediated activation within the parasite to have cytotoxic effects (47). In patients the two compounds require long courses of treatment and exhibit variable efficacy. A number of studies indicated considerable activity of NF and BZ in the acute phase of Chagas disease and reported up to 80% parasitological cures (3, 5). The efficacy of BZ in the treatment of recent chronic disease was demonstrated in Brazil and Argentina, indicating up to 60% parasitological cure in children aged 12 years or less (2, 35). In chronic adult patients with chagasic cardiomiopathy, BZ treatment prevented the progression of the disease and reduced the titer of anti-*T. cruzi* antibodies (reviewed in15, 45, 46).

Taken as a whole, the trials have consistently shown moderate to significant efficacy of BZ in treatment of short-term and long-term chagasic chronic infections. There is consensus that adverse-side effects occur in 30% of patients receiving BZ, but in general the side-effects do not justify cessation of treatment (15).These observations justify WHO recommendation that BZ should be administered to any individual with positive serology for Chagas disease, residing in areas where it is feasible to complete the 60-day treatment under medical supervision (48). In addition, BZ chemotherapy is recommended for congenital and accidental infections, and reactivation of Chagas disease in persons co-infected with HIV (reviewed in 11). On the other hand, in several patients the therapeutic failure of BZ was reported, as evidenced by the presence of circulating parasites and persistence of anti-*T. cruzi* antibodies. Interestingly, an association between drug susceptibility and the geographic areas where the drug was administered was found (reviewed in 30). As a consequence, the therapeutic failure was attributed to biological differences among circulating *T. cruzi* populations (reviewed in 15).

Many studies conducted both *in vitro* and *in vivo* have clearly shown variability of BZ susceptibility in *T. cruzi* strains isolated from different hosts and geographic regions (7, 13, 22, 44). Such phenotypic diversity is not unexpected, in face of the clonal structure of *T. cruzi* population, where clonal lineages have an independent evolution (38). Accordingly, these populations are divergent for many biological and biochemical parameters. On the other hand, on the basis of several genetic markers, *T. cruzi* isolates were separated into two main phylogenetic lineages (36, 37) named as *T. cruzi* I and *T. cruzi* II by a group of experts (4). Some studies demonstrated that *T. cruzi* I isolates were more drug resistant than *T. cruzi* II isolates (39, 40), whereas, others showed a lack of correlation between *in vitro* susceptibility to BZ and the phylogenetic diversity of the parasite (22, 44).

Although several authors attributed the therapeutic failure of BZ and NF, at least in part, to different susceptibilities of *T. cruzi* populations to these drugs, this hypothesis was never demonstrated. Since 1997, one of us (E.D.G.), in conjunction with researchers of the Hospital das Clinicas of the Federal University of Minas Gerais (Brazil), has been conducting a prospective study with 140 patients to test the hypothesis that BZ treatment can impact the evolution of the disease, preventing or retarding the development of defined clinical forms (15). The patients have been clinically, parasitologically and serologically evaluated before and after treatment. A number of patients were considered cured by several criteria, whereas circulating parasites and positive serology for Chagas disease were observed in others, indicating therapeutic failure. In the present study, to investigate whether the therapeutic failure was due to a higher resistance to BZ of the infecting strains, we standardized an *in vitro* test to quantify the susceptibility to this drug. The test was then applied to parasite isolates recovered from patients before and after treatment. We observed that clearance of *T. cruzi* does not correlate with parasite BZ sensitivity. The implications of this observation are discussed.

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