# CENTER FOR DRUG EVALUATION AND RESEARCH

***APPLICATION NUMBER:***

# 209570Orig1s000

# CLINICAL MICROBIOLOGY/VIROLOGY REVIEW(S)

# Division of Anti-Infective Products
# Clinical Microbiology Review

**NDA:** 209570 (SDN-001, -004, -006, -007, -009, -012; -014; -016); Original NDA
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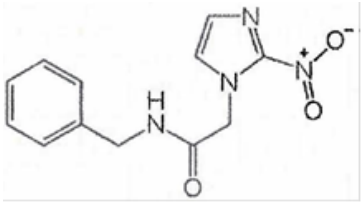
**DRUG PRODUCT NAMES:**
Proprietary name: To be determined
Non-proprietary name: Benznidazole
Chemical name: N-benzyl-2-(2-nitro-1H-imidazol-1-yl) acetamide

**STRUCTURAL FORMULA:**



Molecular weight: 260.25
Molecular formula: $C_{12}H_{12}N_4O_3$

**DRUG CATEGORY:**
Antiparasitic/antiprotozoal

**PROPOSED INDICATION:**
Treatment of Chagas disease.

**PROPOSED DOSAGE FORM, ROUTE OF ADMINISTRATION AND DURATION OF TREATMENT:**
Dosage form: Tablets 12.5 mg and 100 mg
Route of administration: Oral
Dosage: 5 (b) (4) mg/kg/day
Duration: 60 days

**DISPENSED:**
Rx

**RELATED DOCUMENTS:**
PreIND 118976

**REMARKS**

The nonclinical studies *in vitro* and in animals support the activity of benznidazole against different strains and different stages of *Trypanosoma cruzi*. Treatment with benznidazole is effective in improving survival, decreasing parasitic load in blood and tissues including heart, as well as decreasing antibody response to *T. cruzi* antigens. Cure rates varied with the strain of mice and the immune status of the host, the parasite strain as well as the time of initiation of treatment post-infection. Treatment with benznidazole initiated early after infection, especially during the acute phase, appears to be most effective. Decrease in serological response correlates with parasitological cure. In dogs, the suppression of the *T. cruzi* infection with benznidazole treatment during the chronic phase was temporary and effective in reducing systolic cardiac function alterations but not in preventing the development of cardiomyopathy.

The randomized placebo controlled clinical studies suggest that benznidazole treatment for 60 days decreases the proportion of subjects that remain seropositive as well as the antibody titers against *T. cruzi* especially against the recombinant F29 and AT antigens within 1 to 3 years of treatment. The F29 and AT antigens represent antigens from the flagellum of the epimastigotes and trypomastigotes, respectively, of *T. cruzi* and may be a reflection of antibody response to viable parasite.

None of the serological tests, except one, used in the clinical studies reviewed are cleared by the FDA. All the serological assays used for patient enrollment or measuring efficacy were based on detection of IgG antibodies against *T. cruzi* antigens and reflect secondary immune response. The information provided by the Applicant or that available from the referenced publications on details of the methods used and data supporting performance characteristics of the assays in the laboratory where testing of clinical specimens was performed was limited; some of the deficiencies include lack of information on positive and negative controls (calibrators) used, the reproducibility, as well as absence of data to support the cut-offs used to characterize patients as seropositive or seronegative (for more details see consult review memo by Drs Noel Gerald, Kathleen Whitaker, and David Goodwin, Division of Microbiology Devices, CDRH, FDA). Due to limitations of the information available for the performance of the serological assays and the uncertainty about the predictive power of serological results to reliably discriminate which subjects will go on to receive clinical benefit, CDRH recommends serological results should be considered supportive and approval should be based on clinical relevance of serological findings. This is appropriate.

One clinical study, which reported long-term follow-up of patients suggests benznidazole to be effective in decreasing the proportion of patients that changed clinical group to a more severe Kuschnir group as well as those that remained seropositive compared to placebo; similar observation were reported by few other published studies (for more details see clinical review).

It will be useful to conduct a clinical study in asymptomatic subjects, including children, with Chagas disease to confirm the findings observed by the F29-ELISA and AT-CL-ELISA as well as to ascertain the performance of the assays. In addition to the non-conventional ELISAs,

conventional serological tests that are FDA cleared should be used for comparison. Prior to initiation of the clinical study, all details of the methods to be used as well as the performance characteristics of the assay in the laboratory where testing of clinical specimens will be performed should be submitted for review. If possible, a subset of patients that are parasitologically positive at the time of enrollment should be enrolled. This will help compare the serological response in subjects with and without documentation of parasites.

## CONCLUSIONS AND RECOMMENDATIONS

From clinical microbiology perspective, this NDA is approvable pending an accepted version of the labeling (for changes to the labeling please see Section 6.3 of this review).

**Post marketing study**

Conduct a study in asymptomatic subjects, including children, with Chagas disease to confirm the findings observed by the F29-ELISA and AT-CL-ELISA as well as to ascertain the performance of the serological assays.

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**1. EXECUTIVE SUMMARY**

The subject of this NDA is TRADENAME (benznidazole) for the treatment of Chagas disease, a zoonotic tropical parasitic disease, caused by a protozoon, *Trypanosoma cruzi*.

***Mechanism of action***

Benznidazole (BZN), a nitroimidazole, is reduced by a type I nitroreductase (NTR) enzyme of *T. cruzi*. Type I NTR is $O_2$-insensitive and catalyzes the 2-electron reduction of nitroheterocyclic compounds within the parasite producing a series of intermediates/metabolites to yield 4,5-dihydroxyimidazole that slowly dissociate to release glyoxal. The metabolites or released glyoxal may promote damage to macromolecules such as DNA. There is no significant generation of superoxide. Also, BZN inhibits the synthesis of DNA, RNA and proteins. For activity against the intracellular stage (amastigotes) of *T. cruzi* it would be important that the drug cross the host cell membrane.

In mammalian cells, however, BZN is metabolized by reduction of the nitro group to an amino group by a type II NTR, an $O_2$-sensitive enzyme. In the presence of $O_2$, the reduced molecule is rapidly re-oxidized back to the parent compound with the concomitant production of $H_2O_2$ and superoxide anions; the latter can potentially cause oxidative cell damage by generation of reactive oxygen species. A possibility of the activity of BZN due to the production of reactive oxygen species cannot be ruled out. It is unclear if BZN can accumulate in mammalian nucleated cells, where the amastigotes replicate and release trypomastigotes into circulation.

***In vitro activity***

The *in vitro* activity of BZN was reported against the epimastigotes and amastigotes of approximately 51 strains of *T. cruzi* that belong to different distinct typing units (DTUs). The experimental design used in different studies varied; variations include different stages of the parasite and the methods used to measure activity. The activity against the epimastigotes was measured in axenic cultures whereas the activity against the amastigote stage was measured in cultures by infecting mammalian cells such as the Vero cells or murine peritoneal macrophages with the trypomastigote forms; however, different stages of the parasite that include trypomastigotes may be present in cultures depending on the strain. The growth rate of the parasite varied with the strain of the parasite. Time kill studies suggest that the activity of BZN may be concentration and time dependent. The 50% inhibitory concentrations ($IC_{50}$) against the epimastigotes or the amastigotes of the laboratory strains belonging to different DTUs as well as the clinical isolates were ≤19.5 µg/mL. The number of strains belonging to each of the DTUs were small; based on a small number of strains tested, there does not appear to be any difference in the BZN $IC_{50}$s against the different stages as well DTUs of *T. cruzi*. The methods are not standardized and the clinical relevance of *in vitro* sensitivity testing is not known.

***Activity in animal models of T. cruzi***

The activity of BZN was measured in mice, rabbits, and dogs infected with trypomastigotes of approximately 64 strains/clones (a majority of the strains were tested in mice) of *T. cruzi*. Several aspects of *T. cruzi* infection in these animal models mimic human disease. Like humans, parasitemia develops after incubation or a prepatent phase; the patent phase is followed by a subpatent or latent phase. However, the duration of different phases of infection vary with the experimental conditions that include strain of the parasite or immune status of the host. One of the limitations of the murine acute infection models is that unlike humans, infection with *T. cruzi* can be fatal; mice may die within a month post-infection. Mice with chronic infection may survive up to 2 years post-infection. In general, the infection of dogs with *T. cruzi* is not fatal. The immune status of the host plays a role in conferring protection; reactivation has been

reported after immunosuppression during the chronic phase or when parasitemia cannot be detected. Animals may develop cardiac abnormalities that include myocardial fibrosis, and persistence of parasites. The lesions and characteristic chronic pathology, in general, are similar to human disease, and include diffuse myocarditis and signs of heart failure.

The studies in acute infection **murine models** of *T. cruzi* suggest that treatment with BZN is effective in decreasing parasitic load in blood and tissues including heart, improving survival and decreasing antibody response to *T. cruzi* antigens and $P_2\beta$, a member of the acidic ribosomal protein family from *T. cruzi* that have structural similarities with cardiac receptors. Response to treatment varied with the experimental conditions that include strain of mice and the immune status of the host, the parasite strain as well as the time of initiation of treatment post-infection. Treatment with BZN initiated early after infection appears to be most effective. A decrease in serological response correlates with parasitological observations; parasitological cure occurred earlier than seroconversion.

Treatment of mice with BZN during the chronic phase of infection decreased the development of severe chronic cardiomyopathy. There was a reduction in parasite foci, however, there was no significant difference in the fibrotic areas of the hearts between untreated and BZN-treated mice. The parasites were not eradicated. The sera from untreated mice had higher titers of antibodies that recognized peptides from the second extracellular loop of $\beta_1$-adrenergic and M2-muscarinic cardiac receptors. It is possible that the antibodies against *T. cruzi* antigens may exert effects on cross-reactive epitopes on cardiac receptors leading to increased severity of heart dysfunction.

In **rabbits** infected with the Ernestina strain of *T. cruzi*, infections cleared spontaneously within 6 months. Treatment with BZN was effective in reducing the duration of parasitemia during the acute phase. However, myocarditis developed in both treated (in the acute and chronic phase) and untreated rabbits and there was no effect on survival; the authors reported development of lymphomas in all groups that include uninfected rabbits administered BZN (for details see Pharmacology/Toxicology review). Agglutinating antibody titers, measured against the trypsin treated formalin-killed *T. cruzi* culture forms (i.e., antibodies binding the parasite envelope), in treated and untreated rabbits were similar. The reasons for no effect of BZN treatment on antibody response in rabbits compared to mice may be due to different strain of the parasite used for infecting the animals and the assay used for measuring antibody response.

In **dogs** infected with the Colombiana, Y or BE-78 strains, treatment with BZN during the acute phase was effective in reducing parasitemia; the decrease in parasitemia was more in dogs infected with the Y or BE-78 strain compared to the Colombiana strain. All dogs, treated or untreated survived the period of observation. The antibody titers in infected dogs treated with BZN were lower than in untreated dogs against both epimastigote and trypomastigote antigens of the Y strain; the decrease in antibody titers was more in dogs that were parasitologically cured. Lytic antibodies, measured by complement mediated lysis (CoML) of the trypomastigotes of the Y strain of *T. cruzi* were also reduced. There was a difference in antibody response in animals infected with the Y or BE-78 strain of *T. cruzi*. Despite the variability, treatment with BZN reduced IgG, IgG1 and IgG2 levels during the 6 month observation period.

The suppression of the *T. cruzi* (BE-78 strain) infection in dogs, by BZN treatment during the chronic phase, was temporary and effective in reducing systolic cardiac function alterations, but not in preventing the development of cardiomyopathy. A decrease in antibody response was observed, by epimastigote ELISA, in treated dogs compared to the untreated dogs. However, by

the trypomastigote ELISA, such changes in the antibody response were not observed. The CoML antibodies were reduced at Month 24 post-treatment in dogs that were parasitologically cured.

***Drug resistance***

Studies *in vitro* as well as in mice infected with *T. cruzi* suggest a potential for development of resistance to BZN. The *in vitro* studies showed higher $IC_{50}$ values under increasing drug pressure. The growth as well as the flagellar movement of the epimastigotes of the resistant clones, generated *in vitro*, was decreased compared to the parenteral strain. However, there was no difference in the rate of amastigote replication or the extent of infectivity between the parental parasites and any of the drug-resistant clones. The viability of the parasites remained unaltered. The cure rates were lower in mice infected with the resistant clone compared to the parenteral strain. The initial drug exposure is likely to eliminate the sensitive parasites, preserving the resistant ones, which will multiply and dominate the population. The natural and artificial drug pressure can lead to the selection of a subset of the population.

The mechanism of resistance appears to be multifactorial. Studies suggest mutation in the *T. cruzi* nitroreductase (*TcNTR*) gene as well as higher efflux activity of p-glycoprotein (Pgp) and ATP-binding casette (ABC) transporters to be associated with resistance; the higher efflux activity was associated with over-expression of genes (Pgp-$TcPGP_1$ and $TcPGP_2$ genes; ABC transporter- *TcABCG1)* in the resistant line compared to the parental line. Also, some studies reported overexpression of other genes *TcFeSOD-A* and *TcCyP19* that encode superoxide dismutase and cyclophilin, respectively, which are known to be important for biological functions such as signal transduction and may help parasite survival.

***Clinical Microbiology***

The sponsor submitted data for five clinical studies t
(Sosa-Estani *et al*., 1998[1]; de Andrade *et al*., 1996[2]) (b) (4)
(b) (4) The BZN dose used in different studies varied; 5 mg/kg (Radanil product - Sosa-Estani *et al*., 1998[1]; Lafepe product - DNDi-CH-E1224-001), 7.5 mg/kg (Radanil product - de Andrade *et al*., 1996[2]) or 150 mg bid (Lafepe product - Molina *et al*., 2014[3]) BZN for 60 days. The vector control measures were in place, every 6 months, in the dwellings for the two studies in children. (b) (4)

The two studies in children were randomized placebo controlled studies; enrollment of patients was based on ≥3 serological assays. This is in accordance with the Center for Disease Control and Prevention and World health Organization recommendations to strengthen the validity of the

[1] Sosa-Estani S, Segura EL, Ruiz AM, Velazquez E, Porcel BM, and Yampotis C. *Am J Trop Med Hyg* (1998) 59 (4): 526-529.

[2] de Andrade ALSS, Zicker F, de Oliveira RM, Almeida eSS, Luquetti A, Travassos LR, Almeida IC, de Andrade SS, de Andrade JG, and Martelli CMT. Randomised trial of efficacy of benznidazole in treatment of early *Trypanosoma cruzi* infection. *Lancet* (1996) 348: 1407–1413.

[3] Molina I, Gomez I Prat J, Salvador F, Trevino B, Sulleiro E, Serre N, Pou D, Roure S, Cabezos J, Valerio L, Blanco G, Sanchez-Montalva A, Vidal X, and Pahissa A. Randomized trial of posaconazole and benznidazole for chronic Chagas' disease. *New Eng J Med* (2014) 370 (20): 1899-1908.

[4] Viotti R, Vigliano C, Lococo B, Bertocchi G, Petti M, Alvarez MG, Postan M, and Armenti A. Long-term cardiac outcomes of treating chronic Chagas disease with benznidazole versus no treatment. *Ann Int Med* (2006) 144: 724-734.

serology results for diagnosis of patients with Chagas disease. Efficacy was based on serological tests in the two studies in children. In the two studies in adults (Molina *et al*., 2014[3]; DNDi-CH-E1224-001), real-time polymerase chain reaction (RT-PCR) was used, in addition to serology (≥2 tests), for patient enrollment; although serological testing was performed at follow-up visits, efficacy was based on the RT-PCR assay.

The serologic tests that were used in different studies include conventional and non-conventional serological tests:

- **Conventional**: complement fixation test (CFT), indirect hemagglutination assay (IHA), immunofluorescence antibody assay (IFA), and/or enzyme linked immunosorbent assay (ELISA). The antigen used in all these assays was from the epimastigote stage of *T. cruzi*.
- **Non-conventional**: F29-ELISA, AT (antigen trypomastigotes)-chemiluminescence (CL)-ELISA, or recombinant antigen (consisting of 4 epitopes)-ELISA.

  The F29 and AT antigens represent recombinant antigens from the flagellum of the epimastigotes and trypomastigotes, respectively, of *T. cruzi* and the antibody response may be a reflection of response to viable parasites. This is based on published studies that suggest lytic antibodies, measured by a CoML assay, are directed against the live trypomastigotes; the lytic antibodies did not persist in subjects that were negative by xenodiagnosis. Also, the lytic antibodies were detected in mice with live infection but not in those vaccinated with dead parasites.

All the serological tests used in different studies, except one (conventional ELISA from Ortho-Clinical Diagnostics in the study by Molina *et al*., 2014[3]), are not cleared by the FDA. The information provided by the Applicant or that available from the referenced publications on details of the serological or molecular methods used and performance characteristics of the assays in the laboratory where testing of clinical specimens was performed was insufficient for an independent review. Some of the deficiencies include lack of information on positive and negative controls (calibrators) used, reproducibility, limit of quantitation, linearity, as well as absence of data to support the cut-offs used to characterize patients as seropositive or seronegative (for more details see consult review memo by Drs Noel Gerald, Kathleen Whitaker, and David Goodwin, Division of Microbiology Devices, CDRH). Therefore, it could not be ascertained that patients enrolled were seropositive due to an active infection and whether seroconversion post-treatment represents cure. However, the IgG antibody response to *T. cruzi* parasites does reflect that the subjects were exposed to the parasites, and if untreated, are likely to be infected. Also, conventional ELISAs based on the detection of anti-*T. cruzi* IgG antibodies, similar to those used in the benznidazole clinical studies, are cleared by the FDA as an aid for diagnosis.

There is a lot of variability in antibody titers measured by the different serological tests in the four clinical trials. Overall, the results of the two studies in children show a trend towards a decrease in antibody titers as well as the proportion of subjects that became seronegative in BZN treated group compared to the placebo group. However, such differences in the antibody titers or the percentage of subjects that became seronegative were statistically significant only by F29-ELISA (Sosa-Estani *et al*., 1998[1]) and AT-CL-ELISA (de Andrade *et al*., 1996[2]) at Month 12 onwards and at Month 36, respectively, and not by the other serological tests. As the F29 and AT antigens represent antigens from the flagellum, the antibody response may be a reflection of response to viable parasites. A comparison between F29-ELISA or AT-CL-ELISA was not made in any of the study.

In the two studies (Molina *et al*., 2014[3];DNDi-CH-E1224-001) in adults, all subjects remained seropositive, by conventional serological tests, until the end of treatment. By AT-CL-ELISA, in the DNDi-CH-E1224-001study, there is trend towards a decrease in antibody titers at Month 12. Follow-up after Month 12 was not performed. Higher percentage of subjects treated with BZN became RT-PCR negative compared to the control. Some of the patients in the BZN and placebo group that were RT-PCR negative at Day 8, were positive at one or more visits for the duration of the study. It is unclear if RT-PCR positivity after treatment is due to intermittent release of DNA or limitation of the PCR assay. The information supporting validation of the RT-PCR assay, for patient enrollment as well measuring efficacy, did not meet the current scientific and regulatory standards. The results of RT-PCR should be interpreted with caution as a negative PCR result may be indicative only of the absence of circulating DNA; however, the clinical relevance of PCR negative findings is not known.

In one study (Sosa-Estani *et al*., 1998[1]) in children, xenodiagnosis was performed at Months 24 and 48 of follow-up in some of the patients. There was concordance between the results of the serological tests and positive findings by xenodiagnosis. However, there was less concordance between negative findings by xenodiagnosis and the results by different serological tests, including F29-ELISA. Lower concordance between negative findings by xenodiagnosis and serology could be due to lower sensitivity of the xenodiagnosis assay.

The long-term follow-up and clinical relevance was reported in one study in adults (Viotti *et al*., 2006[4]); the results show BZN to be effective in the long-term outcomes in adults with Chagas disease; the proportion of patients that changed clinical group to a more severe Kuschnir group as well as those that remained seropositive were lower in the BZN treated group compared to placebo. Another study (Sosa-Estani *et al*., 2002[5]) reported long-term follow-up, of up to 9 years, of the results of serological testing [IHA, IFA, F29-ELISA and AT immunoenzymatic tests (IET)] of patients treated with BZN. The authors report comparable results by F29-ELISA and AT-IET and state that no significant changes in the electrocardiograms were observed in this population during the follow-up. Similar observations have been reported in other published studies (for more details see clinical review).

The diagnosis of Chagas disease and its pathology are related to the host immune response. In endemic areas, there is a possibility of continuous exposure to infected vectors leading to stimulation of secondary immune response and generation of long-lived memory T-cell and B-cell responses. A possibility of individuals infected with different strains cannot be ruled out. It is unclear if variability in antibody response is due to variability in immune status of the subject, the antibodies produced by different clones of antibody producing cells or response to different antigenic determinants (epitopes) at different time points.

The circulating parasites are scarce or absent in the indeterminate and chronic phases of the disease. There were no studies in subjects with acute phase with documented parasitemia prior to initiation of therapy.

Overall, the studies suggest that BZN treatment decreases antibody titers against *T. cruzi* as well as the number of subjects that remain seropositive, especially by F29- and AT-ELISAs and the

[5] Sosa-Estani S, Herrera de Bravo B, Herrera de Bizzoto L, Canil S, Cura EN, and Segura EL. Evolución serológica a largo plazo en niños infectados por *Trypanosoma cruzi* que cursan fase clínica indeterminada, tratados con benznidazole (2002) http://www fac.org.ar/fec/chagas2/llave/md8/md804/sosaes.htm; English translation provided by the Applicant.

risk for disease progression. As the antibodies can persist for a long time after treatment with BZN, time to seroconversion especially by conventional serological tests, may take over a decade. However, by non-conventional ELISAs, using F29 or AT antigens, seroconversion occurs earlier and may be a reflection of antibody response to a viable parasite.

## 2. INTRODUCTION AND BACKGROUND

The subject of this NDA is TRADENAME (benznidazole) for the treatment of Chagas disease, a zoonotic tropical disease caused by a protozoon, *Trypanosoma cruzi*. About 6 to 7 million adults and children are infected with *T. cruzi* worldwide.[6] Chagas disease is primarily contracted in Latin America, particularly in poor rural areas of Mexico, Central America and South America. Due to international travel and immigration patterns, transmission of Chagas disease to susceptible populations in non-endemic countries such as the United States, Canada and other developed countries is a cause for epidemiologic concern.

There are no FDA approved therapies for the treatment of Chagas disease. The Applicant was granted orphan drug designation and a priority review of this application.

Benznidazole (BZN) has been in clinical use for the treatment of Chagas disease since the early 1970s. Roche Pharmaceuticals obtained the registration of BZN in Brazil, Argentina, Bolivia, Uruguay, Peru, Nicaragua and Japan in the 1970s as Radanil®, Ragonil® or Rochagan®. In the US, BZN is available from the Centers for Disease Control and Prevention for use under investigational protocols for compassionate treatment.

### 2.1. Benznidazole

Following a single 100 mg oral dose of TRADENAME, in healthy volunteers, the average peak plasma concentrations of BZN were between 2.2 and 2.4 µg/mL, and AUC between 45.6 and 50.1 (mg*h/L). The half-life was about 13 hours (for details see the Applicant's proposed labeling).

The serum concentration of BZN (Radanil®) at steady state in 8 patients with Chagas disease treated at a starting dose of 3 mg/kg/day and increased progressively to 5 - 7 mg/kg during the first week, and 7 mg/kg/day to the end of treatment (30 days), ranged between 5.4 and 16.4 µg/mL (Raaflaub, 1980[7]). In another study (Pinazo *et al*., 2013[8]), BZN levels in serum from 54 adult subjects treated with BZN (5 mg/kg/day for 60 days) at Days 15, 30, 45 and 60 of treatment were reported; the mean (standard deviation) concentrations reported were 6.4 (1.9), 6.1 (1.8), 6.2 (2.2) and 5.7 (1.7) µg/mL, respectively.

### 2.2. Biology of *Trypanosoma cruzi*

*T. cruzi* is transmitted primarily to humans and other mammals by blood sucking reduvid (triatomine) bug also known as the kissing bug. In addition to insect bites, *T. cruzi* may also be transmitted through blood transfusions, organ transplantation, breast milk, congenitally and by accidental laboratory exposure.

The trypomastigote (extracellular) forms of *T. cruzi* are transmitted to an uninfected individual when the insect feces are deposited near the open wound of the bite. Inside the host, trypomastigotes invade host cells and differentiate to become amastigotes intra-cellularly. The amastigotes multiply by binary fission and differentiate into trypomastigotes that are released

[6] http://www.who.int/mediacentre/factsheets/fs340/en/

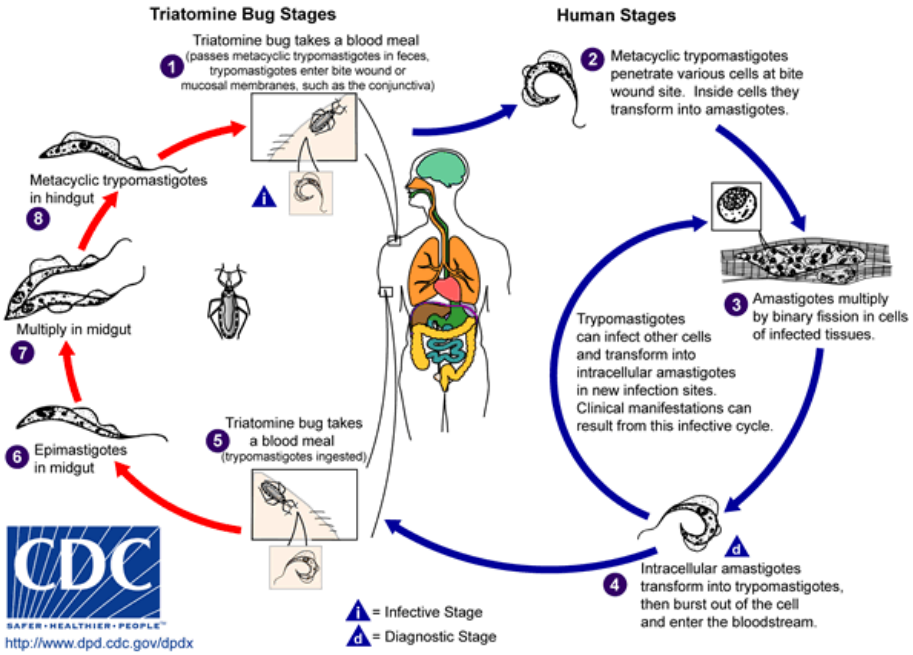
[7] Raaflaub J. Multi-dose kinetics of the trypanosomicide benznidazole in man. *Arzneimittel-Forsch* (1980) 29: 2192-2194.

[8] Pinazo MJ, Guerrero L, Posada E, Rodriguez E, Soy D, and Gascon J. Benznidazole-related adverse drug reactions and their relationship to serum drug concentrations in patients with chronic Chagas disease. *AAC* (2013) 57 (1): 390-395.

into the blood. Trypomastigotes infect cells in a variety of tissues and transform into intracellular amastigotes in different infection sites. Clinical manifestations can result from this infective cycle. The trypomastigotes in blood do not replicate. Replication resumes only when the parasites enter another cell or are ingested by another vector (Figure 1).

The reduvid bug becomes infected by feeding on human or animal blood that contains circulating parasites. The trypomastigotes differentiate in the reduvid bug's midgut to form epimastigotes and multiply. In the hindgut, the parasites differentiate into infective metacyclic trypomastigotes.

Figure 1: Life cycle of *Trypanosoma cruzi* and spectrum of disease



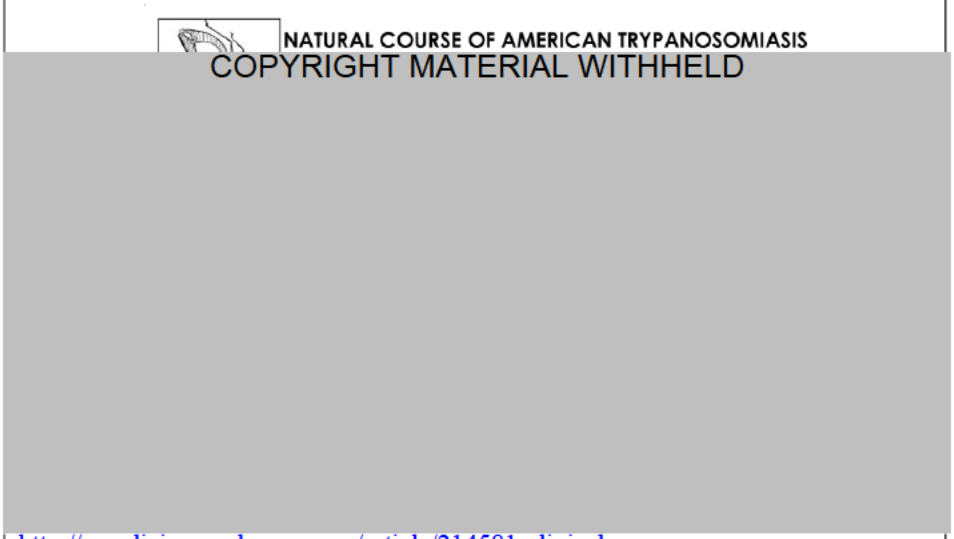

An infected triatomine insect vector (or "kissing" bug) takes a blood meal and releases trypomastigotes in its feces near the site of the bite wound. Trypomastigotes enter the host through the wound or through intact mucosal membranes, such as the conjunctiva ❶. Common triatomine vector species for trypanosomiasis belong to the genera *Triatoma*, *Rhodnius*, and *Panstrongylus*. Inside the host, the trypomastigotes invade cells near the site of inoculation, where they differentiate into intracellular amastigotes ❷. The amastigotes multiply by binary fission ❸and differentiate into trypomastigotes, and then are released into the circulation as bloodstream trypomastigotes ❹. Trypomastigotes infect cells from a variety of tissues and transform into intracellular amastigotes in new infection sites. Clinical manifestations can result from this infective cycle. The bloodstream trypomastigotes do not replicate (different from the African trypanosomes). Replication resumes only when the parasites enter another cell or are ingested by another vector. The "kissing" bug becomes infected by feeding on human or animal blood that contains circulating parasites ❺. The ingested trypomastigotes transform into epimastigotes in the vector's midgut ❻. The parasites multiply and differentiate in the midgut ❼and differentiate into infective metacyclic trypomastigotes in the hindgut ❽.
Source: http://www.dpd.cdc.gov/dpdx/HTML/TrypanosomiasisAmerican.htm

The epimastigotes and trypomastigotes have a long flagellum whereas amastigotes have a short flagellum.

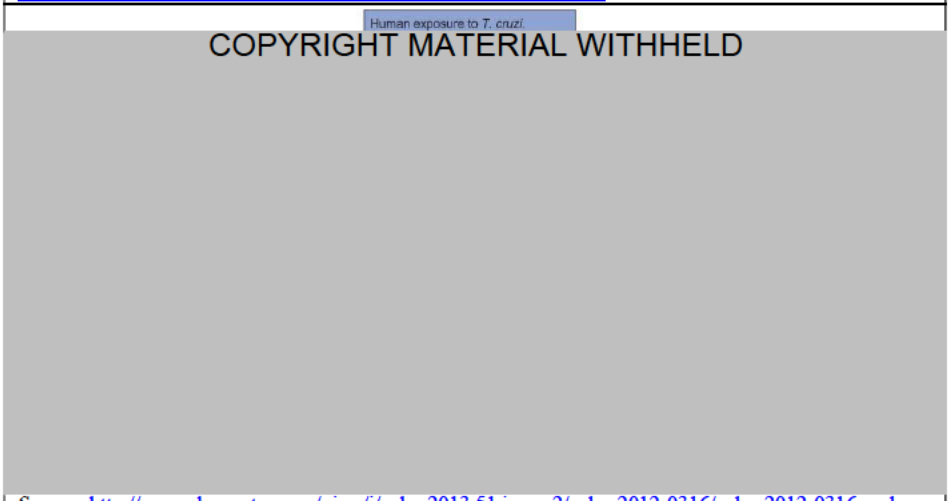
***Clinical Features:***

Chagas disease shows a variable clinical course, ranging from an asymptomatic acute stage to a chronic stage that develops over many years (Figure 2). Persons who live in areas of active transmission are generally continually at risk for exposure to the vectors. The incubation period, (i.e., pre-patent period), before parasites can be detected in blood is thought to vary between one to two weeks. During the acute stage trypomastigotes are present; an intermediate stage is usually asymptomatic with amastigotes, and during chronic stage amastigotes persist.

Figure 2: The spectrum of Chagas disease




http://emedicine.medscape.com/article/214581-clinical




Source: http://www.degruyter.com/view/j/cclm.2013.51.issue-2/cclm-2012-0316/cclm-2012-0316.xml

The **acute phase**, with high parasitemia, lasts for about 2 months. Most cases are asymptomatic. The first sign, in a small percentage of patients, can be a local swelling (skin chancre called chagoma) or unilateral purplish bipalpebral edema (Romaña sign, occurring when inoculation was through the conjunctiva) with local lymphoadenopathies and fever over several weeks. Acute infection may be accompanied by headache, pallor, myalgia, dyspnea, edema in inferior limbs or face, abdominal pain, cough, hepatomegaly, rash, painful nodules, splenomegaly, generalized edema, diarrhea, multiple lymphoadenopathies, rarely myocarditis (chest pain, heart failure) and/or meningoencephalitis (seizures, paralysis). Most symptoms resolve within 4–6 weeks, and patients then enter the indeterminate stage. In the majority of cases, active disease does not proceed further.

Morbidity is higher in children under two, the elderly, immunocompromised patients or in cases with possible high parasitic load, such as seen in oral outbreaks. In patients with AIDS, meningoencephalitis is the most frequent manifestation.

The **chronic phase**, with low or subpatent circulating parasitemia but persistent parasites in target tissues such as myocardium and gastrointestinal nerve ganglia, has different possible clinical forms **(indeterminate and determinate form)**:

- Asymptomatic with normal electrocardiogram (ECG) - **indeterminate form**: Most infected individuals are asymptomatic, with low level of parasitemia which can be intermittent; about 70 - 80 % never develop clinical manifestations of the disease.
- Cardiac form - **determinate chronic Chagas disease**: An estimated 20 - 30% of *T. cruzi*-infected individuals develop cardiomyopathy over the course of their lifetime. Typical features include conduction system disorders, brady- and tachy-arrhythmias, dilated cardiomyopathy, congestive heart failure, apical aneurysm and secondary thromboembolism, including strokes. The infection can lead to sudden death from arrhythmias, heart block, or intractable congestive heart failure.
- Digestive form - **determinate chronic Chagas disease**: This is less common than the cardiac form, and is thought to occur in approximately 10% of those infected in the Southern Cone (Argentina, Bolivia, Uruguay, Paraguay, Chile, Southern Brazil). The main clinical manifestations are dilation of the hollow organs leading to mega esophagus and mega colon.

Advanced congestive heart failure (50- 70%) and sudden cardiac death (30- 50%) are the most common causes of death in patients with chronic disease.

Geographical variations in the frequency of the different clinical forms and in severity have been reported.[9] The clinical manifestations of Chagas disease are due to cell death of the infected cells in the target tissues. In immunocompromised subjects, reactivation can occur and parasites found in the circulation. If left untreated, Chagas disease can be fatal, in most cases due to heart muscle damage.

***Laboratory Diagnosis:***
Laboratory diagnosis of Chagas disease, especially during the acute stage, is by direct observation of the parasite in blood. The most commonly used techniques are blood smear (thick or thin) stained with Giemsa or direct visualization of parasite from a fresh blood sample. Parasite concentration methods, such as the microhematocrit and Strout methods, increase the probability of detecting parasitemia. Isolation/culture of *T. cruzi* can occur by (1) inoculation into animals, (2) hemoculture, or (3) by xenodiagnosis where uninfected triatome nymphs are fed on the patient's blood and the nymph gut or feces are examined for parasites. However, these methods are not very sensitive and not useful during the chronic stage of the disease. Also, the circulating parasites are significantly lower in patients with the intermediate and chronic phase of the disease.

Some of the molecular tests such as nested and real-time (RT) polymerase chain reaction (PCR) have been used to detect *T. cruzi* DNA by amplification of kinetoplast or satellite DNA. However, the assays are limited to research laboratories and considered experimental. None of the nucleic acid based tests are FDA cleared.

Diagnosis during the asymptomatic and chronic stages is based on detection of anti *T. cruzi* antibodies. Various conventional serological tests are available for detecting antibodies against *T. cruzi*. These assays include complement fixation test (CFT), indirect hemagglutination assay (IHA), immuno-fluorescence antibody assay (IFA), radioimmunoassay, and enzyme linked immunosorbent assay (ELISA). Some of these assays are FDA cleared for diagnosis or for screening of blood to be used for transfusions. The Center for Disease Control and Prevention

[9] Zicker F, Smith PG, Almeida Netto JC, Oliveira RM, and Zicker EMS. Physical activity, opportunity for reinfection, and sibling history of heart disease as risk factors for Chagas' cardiopathy. *Am J Trop Med Hyg* (1990) 43: 498–505.

(CDC) and the World Health Organization (WHO) recommend diagnosis of chronic disease by testing with at least two and/or three different serologic tests (Table 1).[10, 11, 12]

Table 1: Laboratory diagnosis of Chagas disease

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Source: WHO Technical Report Series 905 (2002) [11]

Most of the serological assays are based on detection of IgG antibodies against *T. cruzi*. Since circulating antibodies can persist in circulation for a long time, it is often challenging to use serology for diagnosis to reflect active infection or as a marker for cure of the disease after the successful treatment of *T. cruzi* infection. Seroconversion, by conventional serological tests, usually occurs several years after treatment requiring long-term follow-up.

***T. cruzi diversity***

*T. cruzi* is composed of a heterogeneous population of clones circulate among mammalian hosts and vectors.[13, 14] The heterogeneity of the parasite has been extensively studied using biological, biochemical and molecular methods. Earlier, three zymodemes (Z) identified based on isoenzyme types were taken as units of analysis for epidemiological surveys. Based on molecular testing, currently, six discrete typing units (DTUs), named TcI to TcVI, and a seventh one called TcBat have been identified. Some studies propose three clusters (mtTcI, mtTcII, and mtTcIII) based on mitochondrial typing (Barnabé *et al*., 2016[15]; Brenière *et al*., 2016[16]). The phylogenetic diversity of *T. cruzi* genotypes is thought to play an important role in disease pathogenesis (morbidity and mortality) and in approaches towards diagnosis and treatment of

[10] https://www.cdc.gov/parasites/chagas/diagnosis.html

[11] WHO Technical Report Series 905 (2002).

[12] Otani MM, Vinelli E, Kirchoff LV, del Pozo A, Sands A, Vercauteren G, and Sabino EC. WHO comparative evaluation of serologic assays for Chagas disease. *Transfusion* (2009) 49: 1076-1082.

[13] Andrade SG, Magalhes JB, and Pontes AL. Evaluation of chemotherapy with benznidazole and nifurtimox in mice infected with *Trypanosoma cruzi* strains of different types. *Bull World Health Organ* (1985) 63: 721–726.

[14] Coronado X, Zulantay I, Rozas M, Apt W, Sanchez G, Rodriguez J, Ortiz S, and Solari A. Dissimilar distribution of *Trypanosoma cruzi* clones in humans after chemotherapy with allopurinol and itraconazole. *J Antimicrob Chemother* (2006) 58: 216-219.

[15] Barnabé C, Mobarec HI, Jurado MR, Cortez JA, and Brenière SF. Reconsideration of the seven discrete typing units within the species *Trypanosoma cruzi*, a new proposal of three reliable mitochondrial clades. *Infection, Genetics and Evolution* (2016) 39: 176–186.

[16] Brenière SF, Waleckx E, and Barnabé C. Over six thousand *Trypanosoma cruzi* strains classified into discrete typing units (DTUs): Attempt at an inventory. *PLoS Negl Trop Dis* (2016) 10: 1371.

Chagas disease. Although members of all DTUs are capable of causing Chagas disease, the DTUs I, II, V and VI are more often found in humans (Moraes *et al.,* 2014[17]).

## 3. NON CLINICAL MICROBIOLOGY STUDIES

### 3.1. Mechanism of action

Benznidazole, like other nitroimidazoles, is known to be metabolized by nitroreductases. In *T. cruzi*, BZN is reduced to an active form by trypanosomal type I nitroreductase (NTR), a class of enzyme present in many prokaryotes including some of the protozoan parasites but absent from humans. Two enzymes with type I activity have been identified in *T. cruzi*; prostaglandin F2α synthase and nicotinamide adenine dinucleotide, reduced (NADH)–dependent mitochondrial type I NTR. Prostaglandin F2α synthase can mediate 2-electron reduction under anaerobic conditions (Kubata *et al*., 2002[18]). NADH–dependent mitochondrial type I NTR is an $O_2$-insensitive flavin mononucleotide-dependent enzyme that catalyzes the 2-electron reduction of nitroheterocyclic compounds within the parasite, producing toxic metabolites without significant generation of superoxide (Wilkinson *et al*., 2008[19]; 2011[20]; Hall and Wilkinson, 2012[21]). Studies show that BZN, up to 3 mM i.e., 780.8 µg/mL concentration, was not reduced to the nitro anion radical, nor did it stimulate $O_2$ consumption, $O_2$ production and $H_2O_2$ generation by *T. cruzi* cells or microsomal fractions (Moreno *et al*., 1982[22]).

In mammalian systems, BZN is metabolized by reduction of the nitro group to an amino group by a type II NTR, an $O_2$-sensitive enzyme (Bulffer *et al*., 2011[23]). In the presence of $O_2$, the reduced molecule is rapidly re-oxidized back to the parent compound with the concomitant production of superoxide anions and $H_2O_2$ that can potentially cause oxidative cell damage.

Hall and Wilkinson, 2012[21] showed that the trypanocidal activity of BZN mirrored the biochemical screening results i.e., displayed growth-inhibitory properties (Table 2). The metabolism of BZN by the NTR includes a series of intermediates that slowly dissociate to

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[17] Moraes CB, Giardini MA, Kim H, Franco CH, Araujo-Junior AM, Schenkman S, Chatelain E, and Freitas-Junior LH. Nitroheterocyclic compounds are more efficacious than CYP51 inhibitors against *Trypanosoma cruzi*: implications for Chagas disease drug discovery and development. *Nature* (2014) Scientific Reports 4: Article # 4703.

[18] Kubata BK, Kabututu Z, Nozaki T, Munday CJ, Fukuzumi S, Ohkubo K, Lazarus M, Maruyama T, Martin SK, Duszenko M, and Urade Y. A key role for old yellow enzyme in the metabolism of drugs by *Trypanosoma cruzi*. *J Exp Med* (2002) 196: 1241–1251.

[19] Wilkinson SR, Taylor MC, Horn D, Kelly JM, and Cheeseman I. A mechanism for cross-resistance to nifurtimox and benznidazole in trypanosomes. *PNAS* (2008) 105 (13): 5022-5027.

[20] Wilkinson SR, Bot C, Kelly JM, and Hall BS. Trypanocidal activity of nitroaromatic prodrugs: current treatments and future perspectives. *Current Topics Med Chemistry* (2011) 11: 2072-2084.

[21] Hall BS and Wilkinson SR. Activation of benznidazole by Trypanosomal Type I nitroreductases results in glyoxal formation. *AAC* (2012) 56 (1): 115-123.

[22] Moreno SNJ, Docampo R, Mason RP, Leon W, and Stoppani OM. Different behaviors of benznidazole as free radical generator with mammalian and *Trypanosoma cruzi* microsomal preparations. *Archives Biochem and Biophysics* (1982) 218 (2): 585-591.

[23] Bulffer RF, Castro JA, and Fanelli SL. Benznidazole levels in blood vary with age in rats. *Mem Inst Oswaldo Cruz* (2011) 106: 374-377.

release reactive dialdehyde glyoxal that can form adducts with proteins, DNA, and small molecules such as glutathione (Trochine *et al*., 2014[24]).

Table 2: Reduction of nitroimidazoles by trypanosomal type I nitroreductases[a]



A possibility of the reduced metabolites of BZN forming covalent bonding with macromolecules and exhibiting activity against *T. cruzi* has been suggested (Díaz de Toranzo *et al*., 1988[25]; Maya *et al*., 2004[26]). Studies (Coura and de Castro, 2002[27]; Maya *et al*., 2004[26]) show that BZN exerts its trypanocidal effect against all forms of the parasite (intra or extracellular) through reduction to reactive metabolites that likely bind to parasite macromolecules. The effect of BZN on nucleic acid and protein synthesis as well as a possibility of immunomodulatory effects of the drug are summarized below:

- ***Effect on DNA and RNA***

Polak and Richle (1978)[28] reported a decrease in RNA and DNA synthesis measured by incorporation of radiolabeled precursors *in vitro*. Axenic cultures $10^7$ cells/mL containing predominantly epimastigotes of the Y strain were incubated with different concentrations of BZN; cultures were incubated for up to 6 hours with $^{14}C$ labeled uridine or adenine for measuring RNA synthesis and $^{3}H$ thymidine for DNA synthesis. Decrease in RNA and DNA

[24] Trochine A, Creek DJ, Faral-Tello P, Barrett MP, and Robello C. Benznidazole biotransformation and multiple targets in *Trypanosoma cruzi* revealed by metabolomics. *PLoS Negl Trop Dis* (2014) 8**:**e2844. http://dx.doi.org/10.1371/journal.pntd.0002844.

[25] Díaz de Toranzo EG, Castro JA, Franke de Cazzulo BM, and Cazzulo JJ. Interaction of benznidazole reactive metabolites with nuclear and kinetoplastic DNA, proteins and lipids from *Trypanosoma cruzi*. *Experientia* (1988) 44: 880–881.

[26] Maya JD, Rodriguez A, Pino L, Pabon A, Ferreira J, Pavani M, Repetto Y, and Morello A. Effects of buthionine sulfoximine nifurtimox and benznidazole upon trypanothione and metallothionein proteins in *Trypanosoma cruzi*. *Bio Res* (2004) 37: 61-69.

[27] Coura JR, and de Castro, S.L. A critical review on Chagas disease chemotherapy. *Mem Inst Oswaldo Cruz* (2002) 97: 3-24.

[28] Polak A and Richle R. Mode of action of the 2-nitroimidazole derivative benznidazole. *Ann Trop Med Parasitol* (1978) 72 (1): 45-54.

synthesis was concentration and time dependent. A decrease in RNA synthesis was more than DNA synthesis under the experimental conditions tested. Also, the inhibitory effect of BZN on incorporation of adenine was more than that of uridine.

Goijman *et al* (1985)[29] reported that pretreatment of the epimastigotes of the Tulahuen strain of *T. cruzi* with BZN stimulated kinetoplast (k) DNA cleavage. DNA damage was reversible since re-incubation in fresh medium for 24 hours restored electrophoretic and sedimentation patterns to normal.

Rajão *et al* (2014)[30] reported that BZN oxidizes the nucleotide pool; the incorporation of oxidized nucleotides during DNA replication may lead to potentially lethal double-stranded DNA breaks in *T. cruzi* DNA.

- ***Effect on protein synthesis***

Polak and Richle (1978)[28] reported a decrease in protein synthesis measured by incorporation of radiolabeled precursors ($^{14}$C labeled leucine) *in vitro*. Otherwise, the experimental design was similar to that summarized above for the effect on DNA and RNA synthesis. Like for the effect on RNA and DNA synthesis, inhibition of protein synthesis was concentration and time dependent.

Maya *et al*., 2004[26] showed that BZN (2.6 mM i.e., 676.7 µg/mL) decreased the metallothionein (MT) content of the epimastigotes of the Tulahuen strain of *T. cruzi in vitro* (Table 3). MT is a protein that is rich in sulfhydryl groups known to be heat stable and bind cadium.

Table 3: Effect of nifurtimox, BZN upon metallothionein content in *T. cruzi*

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- ***Immunomodulatory effects***

Studies suggest that BZN may activate the immune system. For example, Murta *et al*., 1999[31] reported increased production of IL-12, TNF-α, and reactive nitrogen intermediates *in vitro* using

[29] Goijman SG, Frasch ACC, and Stoppani AOM. Damage of *Trypanosoma cruzi* deoxyribonucleic acid by nitroheterocyclic drugs. *Biochemical Pharmacol* (1985) 34 (9): 1457-1461.

[30] Rajão MA, Furtado C, Alves CL, Passos-Silva DG, de Maura MB, Schamber-Reis BL, Kunrath-Lima M, Zuma AL, Vieira-da-Rocha JP, Garcia JBF, Mendes IC, Pena SDJ, MacedoAM, Franco GR, de Souza-Pinto NC, de Medeiros MHG, Cruz AK, Motta MCM, Teixeira SMR, and Machado CR. Unveiling benznidazole's mechanism of action through overexpression of DNA repair proteins in *Trypanosoma cruzi*. *Environ Mol Mutagen* (2014) 55(4):309-321.

murine macrophages infected with a BZN sensitive strain (Y) of *T. cruzi* in the presence of BZN in comparison to the absence of the drug. However, such an effect was not observed when a clone of the same strain that was resistant to the drug was used for infection. Similar observations were made *in vivo* and *ex vivo*; phagocytosis and destruction of the drug-sensitive parasites were significantly enhanced by drug treatment. *Ex vivo* studies were performed by culture of splenocytes from mice infected with the sensitive or resistant strain of *T. cruzi* and treated with BZN; increased production of nitrite and IFN-γ were reported.

*Comments:*
*Studies show that BZN is reduced by a type I NTR of T. cruzi. The activity of NTR is $O_2$-insensitive and can mediate the 2-electron reduction of nitro drugs through a nitroso, to hydroxylamine derivatives. These can react further to generate nitrenium cations and other highly electrophilic intermediates, which may promote damage to DNA, RNA and proteins. However, in mammalian cells, BZN is metabolized by reduction of the nitro group to an amino group by a type II NTR, an $O_2$-sensitive enzyme. In the presence of $O_2$, the reduced molecule is rapidly re-oxidized back to the parent compound with the concomitant production of superoxide anions and $H_2O_2$ that can potentially cause oxidative cell damage.*

*Studies suggest that BZN may have an immunomodulatory effect.*

### 3.2. Activity *in vitro*

#### 3.2.1. Laboratory strains

The *in vitro* activity of BZN was reported against several strains of *T. cruzi* that belong to different DTUs (Polak and Richle, 1978[28]; Neal and van Bueren, 1988[32]; Revollo *et al*., 1998[33]; Canavaci *et al*., 2010[34]; Moreno *et al*., 2010[35]; Mejia *et al*., 2012[36]; Moraes *et al*., 2014[17]; Franco *et al*., 2015[37]). The experimental design used in different studies varied; variations include

[31] Murta SMF, Ropert C, Alves RO, Gazzinelli RT, and Romanha AJ. In-vivo treatment with benznidazole enhances phagocytosis, parasite destruction and cytokine release by macrophages during infection with a drug susceptible but not with a derived drug-resistant *Trypanosoma cruzi* population. Parasite Immunol (1999) 21(10): 535-544.

[32] Neal RA and van Bueren J. Comparative studies of drug susceptibility of five strains of *Trypanosoma cruzi in vivo* and *in vitro*. *Trans Roy Soc Trop Med Hyg* (1988) 82: 709-714.

[33] Revollo S, Oury B, Laurent JP, Barnabe C, Quesney V, Carriere V, Noel S, and Tabayrenc M. *Trypanosoma cruzi*: impact of clonal evolution of the parasite on its biological and medical properties. *Exp Parasitol* (1998) 89 (1):30-39.

[34] Canavaci AMC, Bustamante JM, Padilla AM, Perez Brandon CM, Simpson LJ, Xu D, Boehlke CL, Tarleton RL. *In vitro* and *in vivo* high-throughput assays for the testing of anti-*Trypanosoma cruzi* compounds. *PLoS Neg Trop Dis* (2010): 4 (7): e740 (www.plosntds.org).

[35] Moreno M, D'ávila DA, Silva MN, Galvão LMC, Macedo AM, Chiari E, Gontijo ED, and Zingales B. *Trypanosoma cruzi* benznidazole susceptibility *in vitro* does not predict the therapeutic outcome of human Chagas disease. *Mem Inst Oswaldo Cruz, Rio de Janeiro* (2010) 105 (7): 918-924.

[36] Mejia AM, Hall BS, Taylor MC, Gómez-PalacioA, Wilkinson SR, Triana-Chávez O, and Kelly JM. Benznidazole-resistance in *Trypanosoma cruzi* is a readily acquired trait that can arise independently in a single population. *JID* (2012) 206: 220-228.

[37] Franco J, Ferreira RC, Ienne S, and Zingales B. ABCG-like transporter of *Trypanosoma cruzi* involved in benznidazole resistance: gene polymorphisms disclose inter-strain intragenic recombination in hybrid isolates. *Infect Genet Evol* (2015) 31:198-208.

different stage of the parasite and the methods used to measure activity (Appendix-1). The activity against the epimastigotes was measured in axenic cultures whereas the activity against the amastigote stage was measured in cultures with mammalian cells such as the Vero cells (a lineage of cells originally isolated from kidney epithelial cells of the African green monkey) and murine peritoneal macrophages. It should be noted that there is a possibility that different stages of the parasite may be present in culture. For example, Polak and Richle (1978)[28] reported about 80% epimastigotes and 20% trypomastigotes in culture whereas Neal and van Bueren (1988)[32] reported the proportion of different stages of the parasite present in culture varied with the strain [Colombiana-100% amastigotes; Sonya clone-50% mixture of epimastigotes and amastigotes; other 3 strains (Tulahuen, Y, and Peru)-100% epimastigotes]. The activity of BZN at the end of incubation period was determined by direct microscopic examination, flow cytometry or by $^{3}$H-thymindine incorporation after incubation for 4 to 5 days in all studies except one by Neal and van Bueren, 1988[32]; incubation of epimastigotes was performed for 4 to 12 days depending on the strain as the growth of different strains varied (for details see Appendix-1). The results show that the 50% inhibitory concentrations ($IC_{50}$s) against different strains (~51), irrespective of the stage of the parasite, were ≤19.5 µg/mL. There is a trend towards higher $IC_{50}$ values against the strains that are considered to be nonresponsive compared to the responsive strains based on studies in animal models (Appendix-1).

Moraes *et al*., 2014[17] reported the *in vitro* activity against tissue-derived trypomastigote forms of a panel of strains belonging to different DTUs. The growth rate of the different strains varied. The Dm28c and Y clones were stated to be more infective, proliferate faster *in vitro*, and less sensitive to BZN compared to the Tulahuen and CL Brener strains; the other 4 strains/clones were stated to be less infective and grow slower (Figure 3). Faster growing strains required higher concentrations of the drug for inhibition. The $IC_{50}$ values were not specified.

Figure 3: The activity of BZN against a phylogenetically broad panel of *T. cruzi*.

Tissue cultures in 384-well plates were infected with tissue-



Richle and Raaflaub (1980)[38] reported that BZN concentrations of 0.4- 0.8 µg/mL inhibit the growth of the parasite whereas 3 - 6 µg/mL of the drug are needed for eliminating both intracellular amastigotes and extracellular trypomastigotes within 4 days. However, the details of the experimental design and results were not included in the publication.

[38] Richle RW and Raaflaub J. Differences of effective antitrypanosomal dosages of benznidazole in mice and man. Chemotherapeutic and pharmacokinetic results. *Acta Topica* (1980) 37: 257-261.

Revollo *et al* (1998)[33] reported the activity of BZN against the different stages of the 21 different stocks that represent 4 major genetic groups (19, 20, 32, and 39) of *T. cruzi*. Epimastigotes ($10^6$/mL), trypomastigotes ($10^7$/mL) and amastigotes (10 parasites:1 Vero cell) were incubated with BZN and the activity of BZN was determined by incorporation of $^3$H-thymidine (epimastigotes and trypomastigotes) or by Giemsa staining of the cells (amastigotes). The BZN $IC_{50}$s were similar against all 3 stages of clones 19 and 20; however, the $IC_{50}$s were slightly lower against the epimastigotes compared to trypomastigotes and amastigotes of clones 32 and 39 (Table 4). Overall, the mean $IC_{50}$s were ≤6.60 µM i.e., 1.72 µg/mL.

Table 4: BZN $IC_{50}$s against 21 different stocks belonging to 4 clones of *T. cruzi*

| **Genetic group (clone)*** | **Stage of the parasite (Mean±SD) µM** | | |
|---|---|---|---|
| | **Epimastigotes** | **Trypomastigotes** | **Amastigotes** |
| 19 (5 stocks) / 20 (5 stocks) | 6.60±1.76 | 6.03±3.52 | 5.41±1.80 |
| 32 (5 stocks) | 1.51±0.51 | 4.97±2.42 | 3.11±1.15 |
| 39 (6 stocks) | 1.80±0.86 | 5.86±3.69 | 2.55±1.21 |
| *Based on 15 isozyme loci; clones 19 and 20 are genetically close to each other. The strain was not specified | | | |

**Time kill studies:**

Three studies (Polak and Richle, 1978[28]; Canavaci *et al*., 2010[34]; Moraes *et al*., 2014[17]) reported results of the time to kill of the epimastigotes and/or amastigotes of the transfected (CL tdTomato) or Y strains of *T. cruzi*. Polak and Richle, 1978[28] reported the activity of BZN to be concentration and time dependent. Higher concentrations killed the parasite in a shorter time; 100 µg/mL killed the parasite in about 24 hours whereas the same effect at lower concentrations of 50 µg/mL and 12.5 µg/mL was achieved at 48 hours and 72 hours, respectively.

Canavaci *et al*., 2010[34] reported the time to kill against both the extracellular epimastigote and intracellular amastigotes stages of the transfected parasite (CL tdTomato) of *T. cruzi*. The results show a concentration dependent decrease in growth of both epimastigotes and amastigotes within 1-2 days of culture with BZN (Figure 4).

Moraes *et al*., 2014[17] reported the activity of BZN against the amastigotes of the Y strain of *T. cruzi*. Exposure of infected cells to a concentration of ≥200 mM i.e., 52.05 mg/mL BZN for at least 72 hours reduced the infection ratios to levels indistinguishable from those of uninfected controls (Figure 5). Two-fold lower concentrations of up to 12.5 mM i.e., 3.25 mg/mL were also nearly 100% effective following 96 hours of exposure. Lower concentrations were less active. The results suggest that the activity of BZN is primarily concentration-dependent.

Figure 4: *In vitro* epimastigote and amastigote growth assays using tdTomato parasites.

**Epimastigote** (A) Epimastigotes growth over



Figure 5: The effect of BZN on growth of the Y strain of *T. cruzi*.

175 U2OS cells (osteosarcoma derived

### 3.2.2. Clinical isolates

Moreno *et al*., 2010[35] reported testing of pre-treatment isolates from 7 patients with indeterminate disease (n=6) or mild cardiac damage (n=1) between the age of 25 and 51 years. The diagnosis was based on two conventional serological tests (IFA and ELISA), antibody-dependent complement-mediated lysis, hemoculture, PCR targeting kDNA in blood samples, and clinical evaluations [ECG, echocardiograms (EKG) and chest X-rays]. Heparinized blood was collected from patients, before and after treatment with BZN, and parasites were isolated by hemoculture for *in vitro* susceptibility testing. The epimastigote stage was predominant in the cultures. The $IC_{50}$ values for isolates collected prior to treatment varied between 15.6 and 51.4 µM i.e., 4.06 and 13.38 µg/mL.

Treatment with BZN (5 mg/kg/day for 60 days) was initiated after hemoculture positive results were obtained. During follow-up, individuals lived in an urban area and did not travel to endemic areas. The post-treatment isolates were collected at different time points. Six of the 7 subjects were hemoculture negative between Months 4 and 112 (Table 5). The *in vitro* susceptibility testing was performed on isolates from 4 patients; the BZN $IC_{50}$s for the post-treatment isolates varied between 10.1 and 55.2 µM i.e., 2.63 and 14.37 µg/mL. There was about 2-fold increase in $IC_{50}$ values for the post-treatment isolates from 2 patients compared to the pre-treatment; there was no change in pre- and post-treatment $IC_{50}$ values for the other 2 patients. The number of isolates tested is very small to compare the effect of treatment on *in vitro* susceptibility to BZN.

Table 5: Patient profile and *in vitro* susceptibility of pre- and post-treatment isolates

A: Patients' profile



B: *In vitro* susceptibility of pre- and post-treatment isolates

*Comments:*
*The methods to measure in vitro sensitivity of different stages of T. cruzi strains to BZN varied and are not standardized. The growth rate of the parasite varied with the strain of the parasite and there is a possibility that the different stages of the parasite present in culture may vary with the strain of the parasite. The results show that the $IC_{50}$ values against the laboratory strains belonging to different DTUs as well as the clinical isolates were ≤19.5µg/mL. The activity against epimastigotes, trypomastigotes or amastigotes forms of the parasite appears to be similar.*

*The $IC_{50}$s against some of the strains are higher than the peak concentrations in serum from subjects with Chagas Disease (for details see Section 2.1 above). It is unclear if BZN can accumulate in phagocytic cells, where the amastigotes replicate and release trypomastigotes into circulation. The activity of BZN against the intracellular stage (amastigotes) of T. cruzi is important as amastigote stage is the predominant form in humans. Also, the killing of the parasite requires that drug cross the host cell membrane.*

*There is a trend towards higher $IC_{50}$ values against the strains that are considered to be nonresponsive compared to the responsive strains based on studies in mice. The nonresponsive strains are more infective and proliferate faster in vitro; strains that are responsive to BZN are less infective and grow slower in vitro. Faster growing strains may require higher concentrations of the drug for inhibition. The number of strains belonging to each of the DTUs was small and the method used in different studies varied. Based on a small number of strains tested, there does not appear to be any difference in the BZN $IC_{50}$ against the different DTUs of T. cruzi. However, the clinical relevance of in vitro sensitivity testing is not known.*

*Time kill studies suggest that the activity of BZN against the epimastigote and amastigote stages of T. cruzi may be concentration and time dependent.*

### 3.3. Activity *in vivo*
The activity of BZN was measured in mice, rabbits and dogs infected with *T. cruzi*.

#### 3.3.1. Mice
In mice, like humans, parasitemia develops after the incubation or prepatent phase; the patent phase is followed by a subpatent or latent phase. However, the duration of different phases of infection may vary with the experimental conditions that include strain of the parasite or immune status of the host.

Studies suggest that some of the immunological, pathological, and physiological aspects of *T. cruzi* infection in mice mimic human disease. However, the limitation is that unlike humans, acute infection can be fatal and mice die within a month post-infection. Mice with chronic infection may survive up to 2 years post-infection. Like humans, immune status of mice plays a role in conferring protection; reactivation has been reported after immunosuppression during the chronic phase or when parasitemia cannot be detected. Mice may develop cardiac abnormalities that include myocardial fibrosis, autoantibody generation, persistence of parasites in deep tissues, or immune response. This may vary with the strain of mice as well as the parasite. Variability in virulence of different parasite strains and resulting parasitemia has been reported.

Several studies reported the activity of BZN in mice with acute (Polak and Richle, 1978[28]; Andrade *et al*., 1985[13]; Neal and van Bueren, 1988[32]; Murta *et al*., 1998[39]; Molina *et al*., 2000[40]; Veloso *et al*., 2001[41]; Camandaroba *et al*., 2003[42]; Urbina *et al*., 2003[43]; Ferraz *et al*., 2007[44]; Canavaci *et al*., 2010[34]; Olivieri *et al*., 2010[45]; Teston *et al*., 2013[46]; Bustamante *et al*., 2014[47]; Francisco *et al*., 2015[48]) and chronic (Molina *et al*., 2000[40]; Garcia *et al*., 2005[49]; Bustamante *et al*., 2014[47]; Francisco *et al*., 2015[48]) infection. The experimental design for the different studies varied; the variations include the strain of mice, the parasite strain, route of infection, the inoculum concentration of trypomastigotes used for infection, time of initiation of treatment post-infection, duration of treatment, the method used for assessing parasitemia and follow-up time points. Approximately, 64 strains and/or clones belonging to different DTUs were tested (for details see Appendix-2).

[39] Murta SMF, Gazzinelli RT, Brener Z, and Romanha AJ. Molecular characterization of susceptible and naturally resistant strains of *Trypanosoma cruzi* to benznidazole and nifurtimox. *Mol and Bio Parasitol* (1998) 93: 203–214.

[40] Molina J, Martins-Filho O, Brener Z, Romanha AJ, Loebenerg D, and Urbina JA. Activities of the triazole derivative SCH 56592 (posaconazole) against drug-resistant strains of the protozoan parasite *Trypanosoma (Schizotrypanum) cruzi* in immunocompetent and immunosuppressed murine hosts. *AAC* (2000) 44 (1): 150-155.

[41] Veloso VM, Carneiro CM, Toledo MJO, Lana M, Chiari E, Tafuri WL, and Bahia MT. Variation in susceptibility to benznidazole in isolates derived from *Trypanosoma cruzi* parental strains. *Mem Inst Oswaldo Cruz, Rio de Janeiro* (2001) 96 (7): 1005-1011.

[42] Camandaroba ELP, Reis AG, Goncalves MS, Reis MG, and Andrade SG. *Trypanosoma cruzi*: susceptibility to chemotherapy with benznidazole of clones isolated from the highly resistant Colombiana strain. *Revista da Sociedade Brasileira de Medicina Tropical* (2003) 36 (2):201-209.

[43] Urbina JA, Payares G, Sanoja C, Lira R, and Romanha J. *In vitro* and *in vivo* activities of ravuconazole on *Trypanosoma cruzi*, the causative agent of Chagas disease. *Int J Antimicrob Agents* (2003) 21 (1): 27-38.

[44] Ferraz ML, Gazzinelli RT, Alves RO, Urbina JA, and Romanha AJ. The anti-*Trypanosoma cruzi* activity of posaconazole in a murine model of acute Chagas' disease is less dependent on gamma interferon than that of benznidazole. *AAC* (2007) 51 (4): 1359-1364.

[45] Olivieri BP, Molina JT, de Castro SL, Pereira MC, Calvet CM, Urbina JA, and Araújo-Jorge TC. A comparative study of posaconazole and benznidazole in the prevention of heart damage and promotion of trypanocidal immune response in a murine model of Chagas disease. *Int J Antimicrob Agents* (2010) 36: 79-83.

[46] Teston APM, Monteiro WM, Reis D, Bossolani GDP, Gomes ML, de Araujo SM, Bahia MT, Barbosa MGV, and Toledo MJO. *In vivo* susceptibility to benznidazole of *Trypanosoma cruzi* strains from the western Brazilian Amazon. *Trop Med and Int Health* (2013) 18 (1): 85-95.

[47] Bustamante JM, Craft JM, Crowe BD, Ketchie SA, and Tarleton RL. New, combined, and reduced dosing treatment protocols cure *Trypanosoma cruzi* infection in mice. *JID* (2014) 209: 150-162.

[48] Francisco AF, Lewis MD, Jayawardhana S, Taylor MC, Chatlain E, and Kelly JM. Limited ability of posaconazole to cure both acute and chronic *Trypanosoma cruzi* infections revealed by highly sensitive *in vivo* imaging. *AAC* (2015) 59 (8): 4653-4661.

[49] Garcia S, Ramos CO, Senra JFV, Vilas-Boas F, Rodrigues MM, Campos-de-Carvalho AC, Ribeiro-dos-Santos R, and Soares MBP. Treatment with benznidazole during the chronic phase of experimental Chagas' disease decreases cardiac alterations. *AAC* (2005) 49 (4): 1521-1528.

### 3.3.1.1. Acute infection model

The studies suggest that BZN is effective in decreasing parasitemia and/or improving survival of infected mice treated during the acute phase of infection. The cure rates varied in different studies which may be due to different experimental conditions used for testing, virulence of different *T. cruzi* strains and host susceptibility. For example, mice infected with the Tulahuen and Peru (characterized as responsive) or Y (characterized as partially responsive) strains were cured whereas mice infected with the Sonya clone or Colombiana strain (characterized as nonresponsive) remained parasitemic (Neal and van Bueren, 1988[32]).

In some of the studies the effect of BZN treatment on clinical features of disease (e.g., cardiac damage) and/or serological response as well as effect of immune status on response to treatment was measured.

#### *3.3.1.1.1. Effect on cardiac damage and splenomegaly*

Olivieri *et al*., 2010[45] reported the activity of BZN in mice infected with the Y strain. BZN was administered between Days 4 and 20 post-infection (PI); heart was collected from a subgroup of animals on Days 8, 15, 35 and 150 days PI and processed for histology and immunochemical evaluations. In the untreated mice, parasites and inflammatory infiltrates were observed as early as 8 days PI and increased with progression of infection. By Day 15, in addition to intense inflammatory infiltrates and parasites, necrosis and fibronectin deposition were observed. Unlike the untreated mice, no inflammation, parasites or fibronectin deposition were observed in the heart tissue from treated mice. The numbers of DAPI-stained nuclei owing to the presence of inflammatory infiltrates were similar in treated and uninfected control mice. The creatine kinase isoform MB levels decreased in BZN treated mice, compared to infected untreated group but were higher than the uninfected control mice. BZN was effective in reducing splenomegaly compared to infected untreated mice; however, the spleen was enlarged compared to the uninfected control mice.

#### *3.3.1.1.2. Effect on serological response*

In some of the studies (Molina *et al*., 2000[40]; Camandaroba *et al*., 2003[42]; Olivieri *et al*., 2010[45]; Veloso *et al*., 2001[41]; Teston *et al*., 2013[46]), serological response was measured (for details see Appendix-2). In most of the studies, the antibody response was measured at the end of study and the findings were used to evaluate parasitological cure. In three studies (Veloso *et al*., 2001[41]; Teston *et al*., 2013[46]; Olivieri *et al*., 2010[45]) the information was available to evaluate the effect of BZN treatment on antibody response in treated and untreated mice and correlate the findings with parasitological cure (for a summary of the experimental design see Appendix-2).

Veloso *et al*., 2001[41] evaluated the antibody response by ELISA (Voller *et al*., 1976[50]). The results showed the presence of antigen specific antibodies in the sera after treatment correlated with the parasitological findings. All mice infected with the Colombiana strain remained parasitemic (PCR and hemoculture) and seropositive up to Day 210 PI (Figure 6). All the mice infected with the Berenice-78 (BE-78) parental strain or BE-78B isolate (collected from a dog 7 years PI) were cured after treatment with BZN; 50% and 70% of the treated mice infected with the BE-78C or BE-78D (collected from dogs 7 and 2 years PI, respectively) isolates were cured. The antibody titers were lower in treated mice compared to untreated mice; the cured mice became seronegative (Figure 7).

[50] Voller A, Bidwell DE, and Bartlett A. Enzyme immunoassays in diagnostic medicine. Theory and practice. *Bull WHO* (1976) *53*: 55-65.

Figure 6: Effect of treatment with BZN in mice infected with parental strains Colombiana, or either of the 2

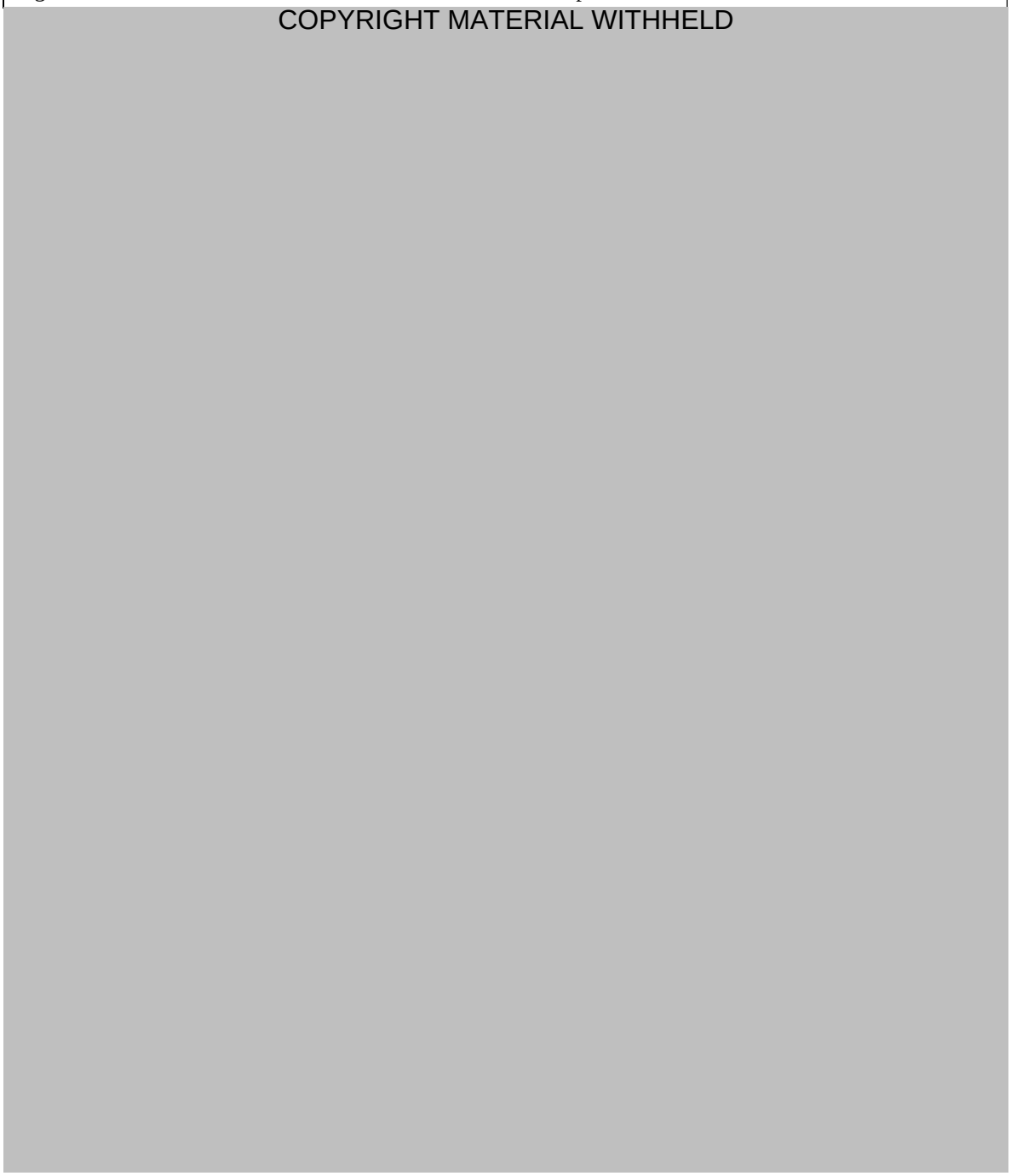


Figure 7: *T. cruzi* specific IgG antibodies in sera of infected control groups and treated mice infected with BE-78 parental strain of *T. cruzi* and their isolates from dogs (BE-78 B, C and D).



B: *In vivo* susceptibility to BZN of parental strain BE-78 and their respective isolates



Teston *et al*., 2013[46] reported the activity of mice infected with 23 different strains belonging to TcI, TcII, or TcIV DTUs of *T. cruzi*. The cure rates varied between 27 and 100%. An increase in the % antibody positive mice was evident in untreated mice infected with the TcI strains but not in those infected with TcII or TcIV strains; this could be due to the fact that a majority of the mice infected with TcII or TcIV strains were antibody positive by Month 3 (Table 6). In the treated group, the antibody positive mice increased from Month 3 to 6; however, the % antibody positive mice in treated group were lower than in the untreated group. Overall, the study suggests that treatment with BZN, compared to the untreated mice infected with different strains, was effective in decreasing the % mice that were parasitemic (by direct examination, hemoculture, or PCR) or antibody positive (by ELISA). However, such differences were statistically significant in the group of mice infected with the Type IV strain (Table 6).

Table 6: Statistical comparisons of the parasitological, molecular and serological parameters among mice treated



PP=patent period

Olivieri *et al*., 2010[45] measured anti-*T. cruzi* IgG antibodies by ELISA using an crude extract of the Y strain of *T. cruzi*; the stage of the parasite used as an antigen was not specified. The authors state that antibody titers increased with the evolution of infection, starting at Day 28; in BZN treated mice, the antibody titers were lower and comparable to uninfected controls (data not shown).

### *3.3.1.1.3. Effect of immune status on response to treatment*

Effect of immunosuppression on response to BZN treatment was evaluated in some of the studies. Molina *et al*., 2000[40] compared the activity of BZN in immunocompetent and immunosuppressed mice infected with the CL, Y, or Colombiana strains. The results showed lower survival rate and survival time in immunosuppressed mice compared to immunocompetent mice.

Ferraz *et al*., 2007[44] reported that IFN-γ and IL-12 knockout (KO) mice were more susceptible to *T. cruzi* infection and less responsive to BZN treatment compared to the WT mice. Also, reactivation occurred within 2 to 14 days of discontinuation of treatment. Similar observations were made by Romanha *et al*., 2002[51] in C57BL/6 IFN-γ KO, IL-12 (protein 40) KO, p55–tumor necrosis factor receptor (TNFR) KO, and inducible nitric oxide synthase (iNOS) KO mice - cure rates were lower (0%, 58%, 65%, and 72%, respectively) compared to the WT mice infected with the trypomastigotes of the Y-strain of *T. cruzi* and treated with BZN. All the IL-4 KO mice were parasitologically cured (Table 7). The results suggest a role of immune response in

[51] Romanha AJ, Alves RO, Murta SM, Silva JS, Ropert C, and Gazzinelli RT. Experimental chemotherapy against *Trypanosoma cruzi* infection: essential role of endogenous interferon-gamma in mediating parasitologic cure. *J Infect Dis* (2002) 186: 823–828.

conferring protection. It is possible that activation of the immune system by the parasite and endogenous interferon-γ play a role in the activity of BZN against *T. cruzi* infection.

Table 7: Parasitological cure rates among wild-type and knockout (KO) mice infected with *Trypanosoma cruzi* and treated with BZN.



### 3.3.1.2. Chronic infection model

Several studies (Molina *et al*., 2000[40]; Garcia *et al*., 2005[49]; Bustamante *et al*., 2014[47]; Francisco *et al*., 2015[48]) reported the activity of BZN in mice with chronic infection; 7 strains of *T. cruzi* were tested. Like for the studies in acute infection models, there is a lot of variability in the experimental design. The inoculum concentration of the parasites was lower than that used for the acute infection models (for details see Appendix-2). BZN treatment was initiated when mice were in the latent phase of infection i.e., parasitemia was undetectable. Overall, the studies suggest that BZN was effective in improving survival and suppressing parasitemia.

In some of the studies, the effect of BZN treatment on clinical features of disease and serological response was measured.

#### *3.3.1.2.1. Effect on ECG and histological parameters*

Garcia *et al*., 2005[49] evaluated the effect of BZN treatment on heart function in mice infected with the Colombiana strain. Treatment was initiated on Day 45 PI for 1 week followed by weekly administration for 8 months. A reduction in the detectable parasite foci (3-fold) as well as inflammatory cells (2-fold) was observed in the hearts of mice treated with BZN compared to the untreated mice at Month 10 PI (Figures 8A and 8B). Although both untreated and treated infected mice had significant alterations in their electrocardiograms (ECGs) compared to those of the healthy mice, only 20% of the BZN treated mice had cardiac conduction disturbances including intraventricular conduction disturbances, atrioventricular blocks, and extrasystoles compared to untreated infected mice. There was no significant difference in the fibrotic areas of the hearts between untreated and BZN-treated mice (Figure 8C).

Figure 8: Decreased parasitism and inflammation in the hearts of BZN-treated and untreated mice



***3.3.1.2.2. Effect on serological response***

Garcia *et al*., 2005[49] reported that the antibody titers against the extract of the epimastigotes of *T. cruzi* and an immunodominant TS (an enzyme abundant on the *T. cruzi* parasite surface) antigens were lower in the sera from BZN-treated mice compared to the sera from untreated mice (Figure 9).

Figure 9: Antibodies levels, by ELISA, against (A) epimastigote extract and (B) an immunodominant (TS) antigen in sera from mice infected with the Colombiana strain of *T. cruzi*.



Antibody titers against $P_2\beta$ (a member of the acidic ribosomal protein family from the *T. cruzi* parasite with structural similarities with cardiac receptors) and trans-sialidase as well as antibodies against the peptides of the second extracellular loops of β1-adrenergic and $M_2$-muscarinic cardiac receptors were lower in the sera from BZN-treated mice compared to the sera from untreated mice.

*Comments:*
*Overall, the studies suggest that treatment with BZN is effective in decreasing parasitic load in blood and tissues including heart, improving survival and decreasing antibody response to T. cruzi antigens and $P_2\beta$. Response to treatment varied with the strain of mice as well as the parasite, the immune status of the host, and time of initiation of treatment PI. Treatment with*

*BZN initiated early after infection appears to be most effective. Although treatment with BZN in the chronic phase of infection prevents the development of severe chronic cardiomyopathy the parasites are not eradicated.*

*The sera from untreated mice had higher titers of antibodies that recognized peptides from the second extracellular loop of $\beta_1$-adrenergic and M2-muscarinic cardiac receptors. It is possible that the higher antibody titers participate in the increased severity of heart dysfunction in untreated mice, as antibodies against T. cruzi antigens may exert effects on cross-reactive epitopes on cardiac receptors and modulate the function of the heart.*

### 3.3.2. Rabbits

Similar to mice, experimental conditions such as variability in parasite strains affect the course of development of infection and resulting parasitemia in rabbits. No ECG abnormalities occur during the acute phase; however, during the chronic phase symptoms of cardiac involvement have been reported. The lesions and characteristic chronic pathology is similar to human disease, and includes diffuse myocarditis and signs of heart failure.

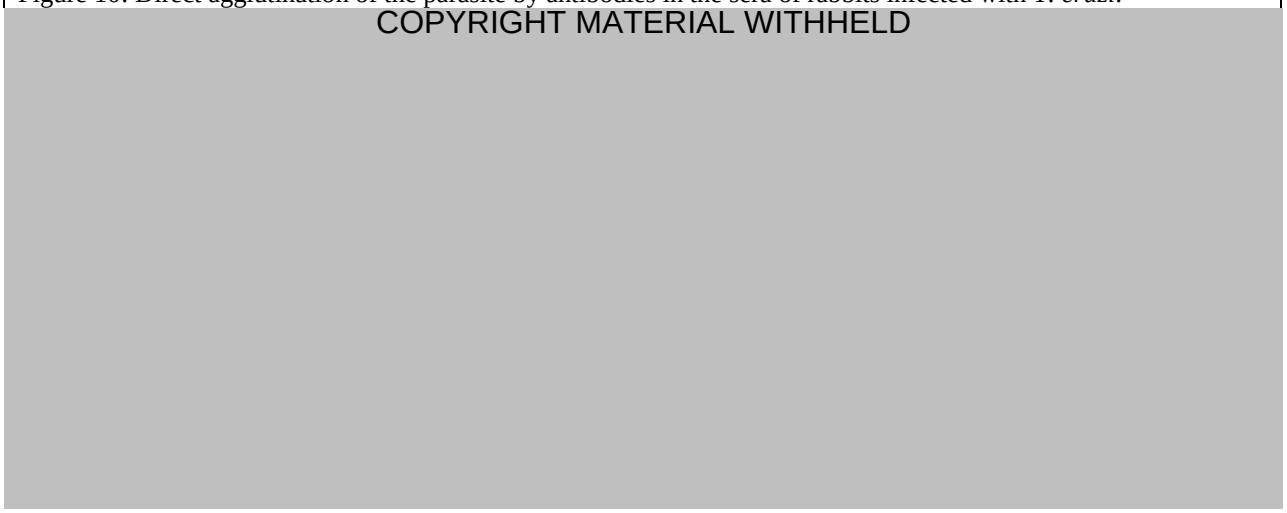
Teixeira *et al* (1990a[52]; 1990b[53]) reported the activity of BZN in New Zealand white rabbits infected subcutaneously with $10^6$ of trypomastigotes/kg body weight of the Ernestina strain of *T. cruzi*. Parasitemia was monitored by xenodiagnosis and repeated every 15 days for 6 months and every month thereafter. Treatment with BZN (8 mg/kg/day for 60 days, IP) was initiated during the **acute phase** (Month 2 PI) or the **chronic phase** (Month 6 PI). The peak parasitemia in all rabbits was reached at Day 45 post- infection; at that point, ~35% of the reduviid bug (*Dipetalogaster maximus*) fed on infected rabbits became infected with *T. cruzi*. Compared to untreated rabbits, treatment with BZN reduced the duration of parasitemia by 75%. However, there does not appear to be any effect on survival (for details see Appendix-2); this may be due to the development of lymphomas in infected and uninfected rabbits administered BZN; lymphomas developed in the 3 of the 6 uninfected treated rabbits as well.

Agglutinating antibodies against the trypsin treated formalin-killed *T. cruzi* culture forms i.e., antibodies binding the parasite envelope were detected 15 days PI; the antibody titers increased by Month 2 and remained high until Month 20 PI in BZN treated (acute or chronic phase) and untreated rabbits (Figure 10).

[52] Teixeira ARL, Silva R, Neto EC, Santana JM, and Rizzo LV. Malignant, non-Hodgkin's lymphomas in *Trypanosoma cruzi*-infected rabbits treated with nitroarenes. *J Comp Path* (1990)103: 37-48.

[53] Teixeira ARL, Cordoba JC, Maior IS, and Solorzano E. Chagas' disease: lymphoma growth in rabbits treated with benznidazole. *Am J Trop Med Hyg* (1990b) 43 (2): 146-158.

Figure 10: Direct agglutination of the parasite by antibodies in the sera of rabbits infected with *T. cruzi*.




*Comments:*
*In rabbits infected with the Ernestina strain of T. cruzi, infections cleared spontaneously within 6 months. Treatment with BZN was effective in reducing the duration of parasitemia during the acute phase. However, myocarditis developed in both treated (in the acute and chronic phase) and untreated rabbits and there was no effect on survival; this may be due to the development of lymphomas in all groups of rabbits treated or untreated.*

*The antibody response in treated and untreated rabbits was similar. The reasons for no effect on antibody response in rabbits compared to mice are unclear. It was noted that the strain of the parasite used for infection or the assay used for measuring antibody response were different.*

### 3.3.3. Dogs

Dogs are an important reservoir of *T. cruzi* parasites. The clinical findings and immunopathogenic mechanisms are similar to those reported in patients with Chagas disease. In general, the infection with *T. cruzi* is not fatal.

Several published studies by the same group (Guedes *et al*., 2002[54]; de Figueiredo Diniz *et al*., 2010[55]; Santos *et al*., 2012[56] and Santos *et al*., 2016[57]) reported the activity of BZN in Mongrel dogs infected with either the Colombiana, Y or BE-78 strains of *T. cruzi*. The results show that

[54] Guedes PMM, Velosa VM, Tafuri L, Galvao LMC, Carneiro CM, Lana M, Chiari E, Soares KA, and Bahia MT. The dog as model for chemotherapy of the Chagas' disease. *Acta Tropica* (2002) 84: 9-17.

[55] de Figueiredo Diniz L, Caldas IS, Guedes PMM, Crepalde G, de Lana M, Carneiro CMM, Talvani A, Urbina JA, and Bahia MT. Effects of ravuconazole treatment on parasite load and immune response in dogs experimentally infected with *Trypanosoma cruzi. AAC* (2010) 54 (7): 2979-2986.

[56] Santos FM, Lima WG, Gravel AS, Martins TAF, Talvani A, Torres RM, and Bahia MT. Cardiomyopathy prognosis after benznidazole treatment in chroniccanine Chagas' disease. *J Antimicrob Chemother* (2012) 67: 1987–1995.

[57] Santos FM, Mazzet AL, Caldas S, Goncalves KR, Lima WG, Torres RM, and Bahia MT. Chagas cardiomyopathy: The potential effect of benznidazole treatment on diastolic dysfunction and cardiac damage in dogs chronically infected with *Trypanosoma cruzi*. *Acta Tropica* (2016) 161: 44-54.

treatment with BZN during the acute or chronic phase of *T. cruzi* infection was effective in reducing parasitic load in the blood or heart tissue measured by direct microscopic examination, hemoculture, and PCR; however, the response varied with the parasite strain (for details see Appendix-2). In some of the studies the effect of BZN treatment on clinical features of disease and serological response was reported.

**3.3.3.1. Acute infection model**

Mongrel dogs were infected IP with $2x10^3$ trypomastigotes/kg of Colombiana, Y or BE-78 strain. BZN (7 mg/kg bid) treatment was initiated immediately after dogs became parasitemic (12 to 22 days PI) for 45 or 60 days (Guedes *et al*., 2002[54]; de Figueiredo Diniz *et al*., 2010[55]). Parasitemia was followed at different time intervals by fresh blood examination, hemoculture, and PCR assay. Antibody response was measured at different time intervals by complement-mediated lysis (CoML) of the trypomastigotes of the Y strain as well as ELISA. ELISA was performed using antigen from the epimastigotes and trypomastigotes of the Y strain. The animals were considered cured when all the tests, including serological tests, were negative.

All the dogs survived the period of observation. All untreated control animals were hemoculture and PCR positive at Month 6. In one study (Guedes *et al*., 2002[54]) the response to treatment varied with the strain; for example, one dog infected with the Colombiana strain was hemoculture positive and all the 4 dogs were PCR positive. A majority of the dogs infected with the BE-78 strain were hemoculture negative at Month 6 post-treatment; one dog remained PCR positive. All four dogs infected with the Y strain were hemoculture and PCR negative (Table 8).

In another study (de Figueiredo Diniz *et al*., 2010[55]), BZN was shown to be effective in suppressing parasitemia (measured by culture and PCR) in dogs infected with either the Y or BE-78 strains and all animals survived (Table 8).

Table 8: Parasitological and molecular tests in blood of infected dogs with *T. cruzi* BE-78, Y or Colombian strains and treated in acute phase (12-22 days post-infection) with 7 mg of BZN for 45 – 60 days

Guedes *et al*., 2002[54] – treatment for 45 days

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de Figueiredo Diniz *et al*., 2010[55] - treatment for 60 days; parasitic load after 30 days of treatment

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In some studies, the effect of BZN treatment on clinical features of disease and serological response was reported.

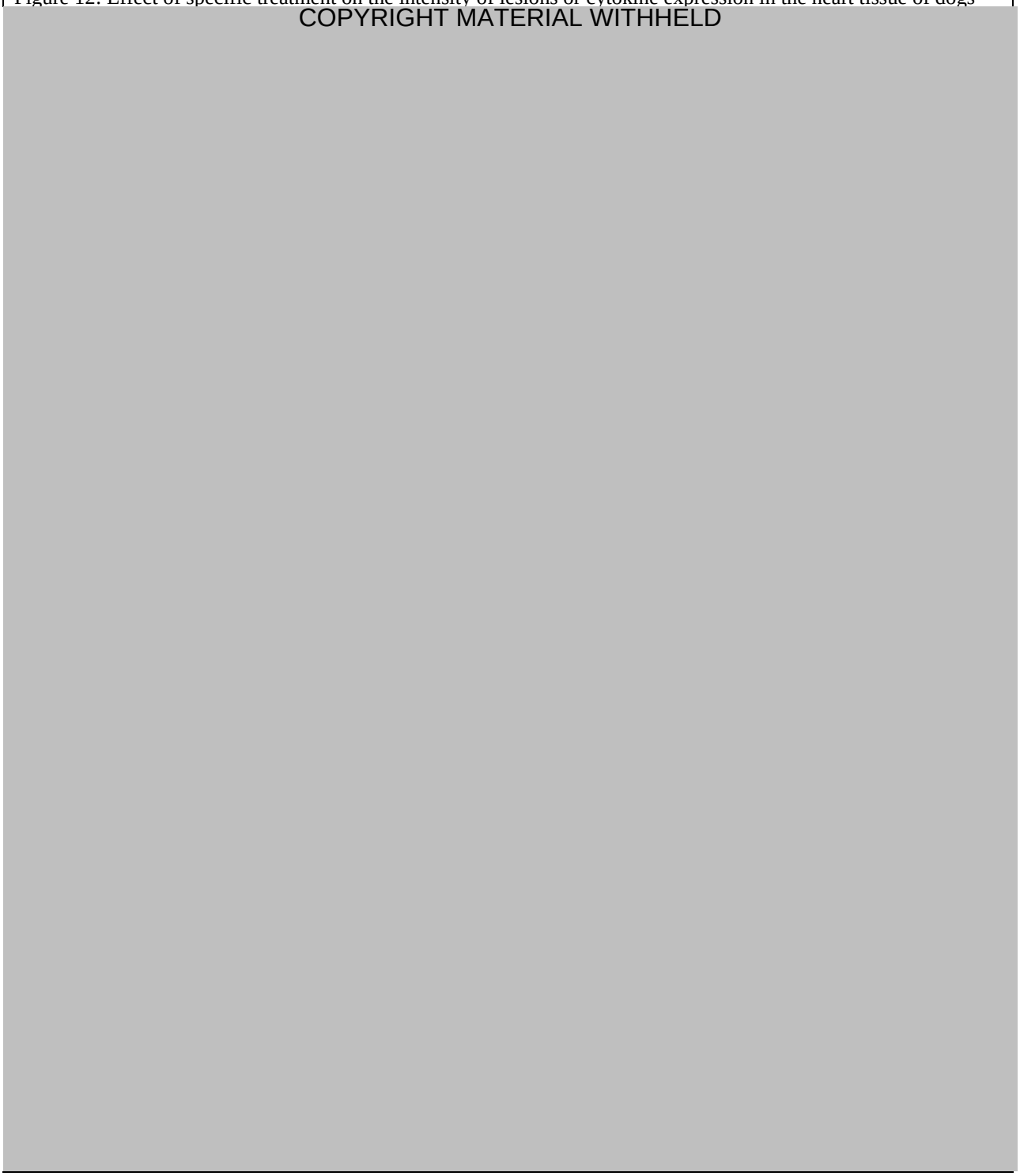
#### *3.3.3.1.1. Effect on ECG and histological parameters*

de Figueiredo Diniz *et al*., 2010[55] reported the effect of BZN treatment on histopathological evaluation of heart tissue at Month 6 after the end of treatment. BZN was effective in reducing the inflammatory and fibrotic cardiac lesions in dogs infected with either the Y or the BE-78 strain. Also, IFN-γ expression was reduced in the heart tissue of treated dogs infected with the Y or the BE-78 strains compared to the untreated control group (Figures 11and 12). However, IL-10 expression was different; IL-10 levels were higher in treated dogs, compared to untreated dogs, infected with the Y strain and same as the control group in dogs infected with the BE-78 strain.

Figure 11: Effect of specific treatment on the intensity of lesions or cytokine expression in the heart tissue of dogs

Figure 12: Effect of specific treatment on the intensity of lesions or cytokine expression in the heart tissue of dogs





***3.3.3.1.2. Effect on serological response***

The effect of BZN treatment on serological response was reported in two studies (Guedes *et al*., 2002[54]; de Figueiredo Diniz *et al*., 2010[55]). Guedes *et al*., 2002[54] reported high levels of anti-*T. cruzi* IgG antibodies against the epimastigote and trypomastigote antigens of the Y strain, measured by ELISA, in untreated dogs around Day 20 PI; the antibody titers remained high for the duration of the study (Figure 13). The antibody titers in infected dogs treated with BZN were lower than in untreated dogs against both epimastigote and trypomastigote antigens of the Y

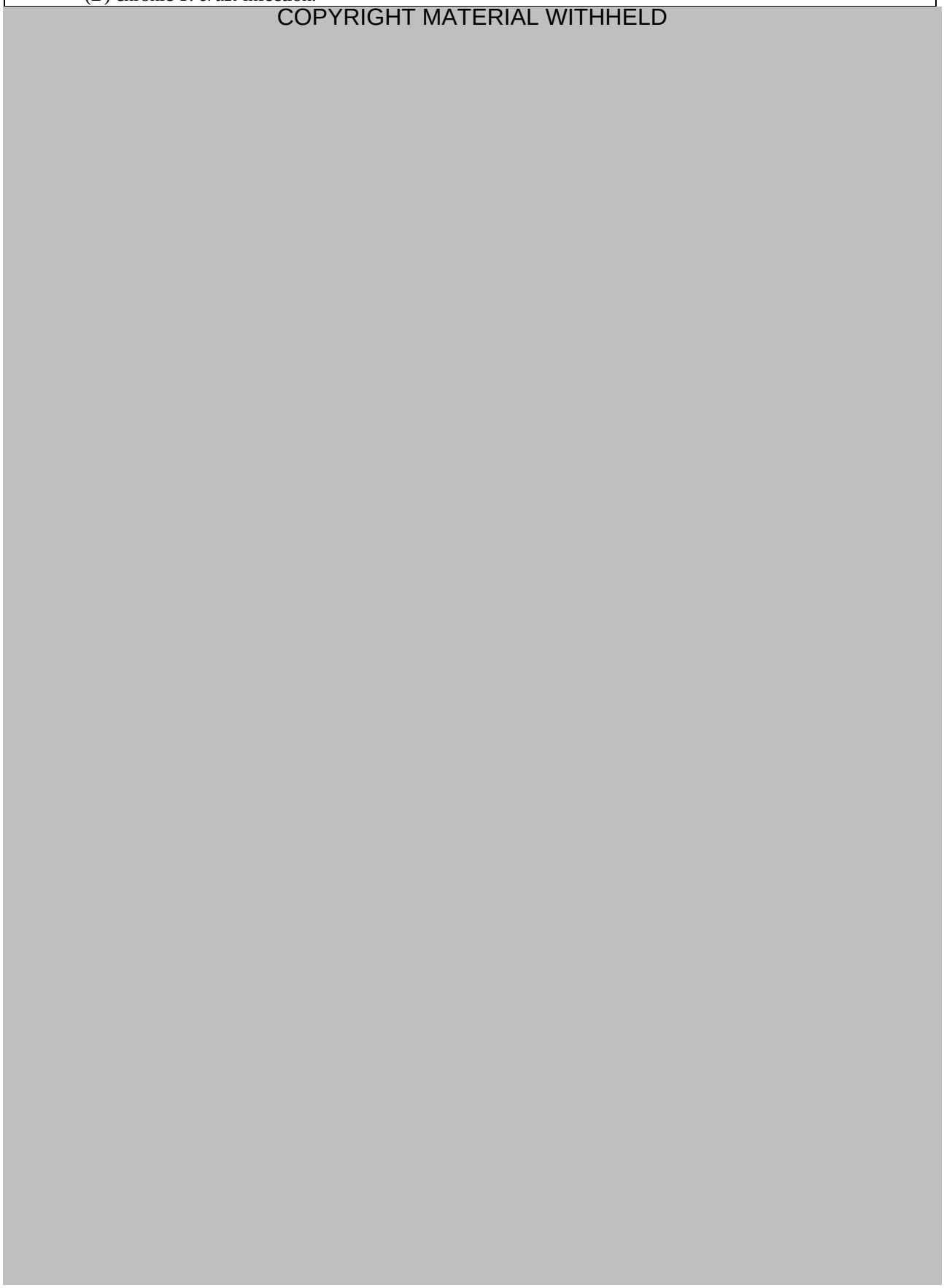
strain as well as the CoML antibodies; the decrease was more in dogs that were parasitologically cured (Figure 13). All the 4 dogs infected with the Colombiana strain (considered to be nonresponsive) and one dog infected with the BE-78 strain (considered to be responsive), remained antibody positive. All the dogs that were negative by ELISA-epimastigote were also negative by CoML.

All the untreated infected dogs were antibody positive by the CoML assay. Of the treated dogs, 5 dogs (1 infected with the BE-78 and 4 with the Colombiana strain) were antibody positive at Month 6 post-treatment (Figure 13).

Based on hemoculture, PCR and serologic (ELISA-epimastigote and CoML) findings, 68.8% (11/16) of the treated animals were cured.

Figure 13: Serological response by ELISA and complement mediated lysis of the Y strain in dogs with (A) acute and (B) chronic *T. cruzi* infection.





In another study by the same group (de Figueiredo Diniz *et al*., 2010[55]), the anti-*T. cruzi* IgG, IgG1, and IgG2 antibody titers were measured monthly, by ELISA using epimastigote antigen from the Y strain. The results show that in infected untreated dogs, antibody titers increased until about the 90th day of infection and stabilized afterwards until the end of the experiment. IgG1 levels were higher in dogs infected with the BE-78 strain compared to the Y strain of *T. cruzi*. Compared to the untreated dogs, treatment with BZN reduced IgG, IgG1 and IgG2 levels during the 6 month observation period (Figure 14).

Figure 14: Antibody (IgG, IgG1, and IgG2) levels in the sera of dogs infected with the Y or BE-78 strains of *T. cruzi*.



### 3.3.3.2. Chronic infection model

Three studies (Guedes *et al*., 2002[54]; Santos *et al*., 2012[56]; 2016[57]) reported the activity of BZN in Mongrel dogs with chronic *T. cruzi* infection. The experimental design was same as above for

the acute infection model, except that dogs were infected with the BE-78 strain and the treatment was initiated on Day 100 PI. The results show that 1 dog remained hemoculture positive and 5 of the 8 dogs were PCR positive at Month 6 (Table 9). The two untreated dogs were parasitologically positive.

Table 9: Parasitological and molecular tests in blood of infected dogs with *T. cruzi* BE-78 strain and treated in chronic phase with 7 mg of BZN for 45 days



Santos *et al* (2012[56]; 2016[57]) reported that BZN treatment was effective in reducing parasitemia, measured by PCR (kinetoplast DNA) in blood at Month 1 post-treatment; however, this was followed by an increase in parasitemia at Month 12 post-treatment (Figure 15).

Figure 15: Influence of BZN treatment on the parasite load.



The effect of BZN treatment on clinical features of disease (cardiac function) and serological response was reported.

### *3.3.3.2.1. Effect on ECG and histological parameters*

Santos *et al* (2012[56]; 2016[57]) reported that BZN treatment was effective in reducing parasite load in heart at Month 1 post-treatment compared to untreated animals; this was associated with an improvement in systolic heart function. However, at Month 12 post-treatment, there was an increase in parasitic load in the blood and heart tissue as well as cardiac dysfunction. These changes were associated with an increase in IL-10 levels and no change in TNF-α levels at Month 1 in both infected untreated and BZN treated dogs compared to uninfected dogs. However, at Month 12 the elevation of TNF-α synthesis and the reduction in IL-10 synthesis by PBMCs were not altered by BZN treatment. An immunological imbalance of TNF-α and IL-10 synthesis by PBMCs, with high levels of TNF-α and low levels of IL-10 may contribute to the progression of cardiomyopathy disease.

The parasitic load reduction after BZN treatment was associated with the lower degree of tissue damage in animals euthanized at Month 1 post- treatment; this was followed by an increased cardiac damage, reaching levels similar to those in untreated animals at the one-year follow-up. Overall, the results suggest that the temporary reduction in the parasitic load induced by BZN treatment was not effective in preventing myocardial lesions and diastolic dysfunction for long after treatment.

***3.3.3.2.2. Effect on serological response***

Guedes *et al*., 2002[54] reported that the antibody response by the epimastigote ELISA was different between treated and controls animals (Figure 13). A decrease of antibody titers was greater in treated dogs compared to the untreated dogs. However, by the trypomastigote ELISA, such changes in the antibody response were not observed (Figure 13). The lytic antibodies were present in all animals, treated and untreated, at Month 9 post-treatment; however, at Month 24, the CoML antibodies were decreased in the dogs that were cured compared to the untreated dogs (Figure 13). At Month 24 post-treatment, three dogs that were hemoculture, PCR and antibody negative by epimastigote ELISA, became CoML negative.

By hemoculture, PCR and serological tests, 3 of the 8 dogs were considered cured.

*Comments:*
*Overall, the studies suggests that treatment with BZN during the acute phase is effective in reducing parasitemia in Mongrel dogs infected with the Colombiana, Y or BE-78 strains; the decrease in parasitemia was more in dogs infected with the Y or BE-78 strain compared to Colombiana strain. All dogs, treated or untreated survived the period of observation. The antibody titers in infected dogs treated with BZN were lower than in untreated dogs against both epimastigote and trypomastigote antigens by ELISA as well as CoML against the Y strain; the decrease was more in dogs that were parasitologically cured. There was a difference in antibody response in animals infected with the Y or BE-78 strain of T. cruzi. Despite the variability, treatment with BZN reduced IgG, IgG1 and IgG2 levels during the 6 month observation period.*

*Suppression of the T. cruzi (BE-78 strain) infection by BZN treatment during the chronic phase was temporary and effective in reducing systolic cardiac function alterations, but not in preventing the development of cardiomyopathy. A decrease in antibody response was observed, by epimastigote ELISA, in treated dogs compared to the untreated dogs. However, by the trypomastigote ELISA, such changes in the antibody response were not observed. The COML were present in all animals, treated and untreated, at Month 9 post-treatment but reduced at Month 24 especially in dogs that were cured.*

## 3.4. Drug Resistance

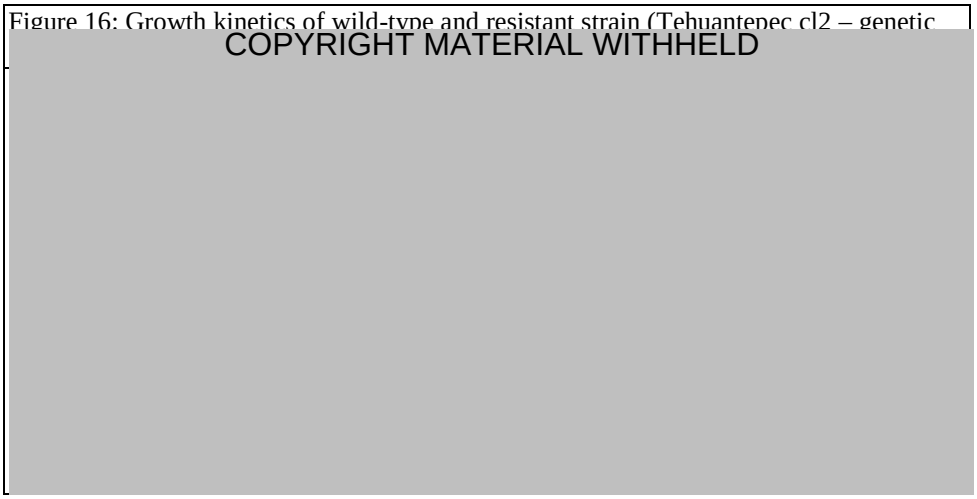
### 3.4.1. Drug resistance *in vitro*

#### 3.4.1.1. Potential for development of drug resistance

A potential for develop of resistance to BZN was measured by comparing the growth kinetics of different strains and an increase in $IC_{50}$ values under increasing drug pressure.

Villarreal *et al*., 2005[58] compared the growth kinetics of different strains among representative *T. cruzi* stocks exposed to BZN by flow cytometry. Briefly, cloned epimastigotes of different strains were grown in L30TC culture medium [liver infusion tryptose (60%) and TC-100 (30%)] with 10% FCS and different concentrations of BZN (15, 50, 100, and 200 µM i.e., 3.9, 13.0, 26.0, and 52.0 µg/mL). The growth rate was lower for all the drug-resistant epimastigotes than for the WT; the growth rate, decreased as the drug resistance increased (Figure 16). The doubling time was as high as 35 hours in the presence of 200 µM i.e., 52.0 µg/mL BZN, the maximum concentration tested in this study. The viability of the parasites remained unaltered.

Figure 16: Growth kinetics of wild-type and resistant strain (Tehuantepec cl2 – genetic




The authors stated that some morphological changes as well as a decrease in flagellar movement of resistant parasites were observed compared to the WT parasites (unpublished data).

Mejia *et al*., 2012[36] reported approximately 10-fold increase in BZN $IC_{50}$ against the epimastigotes of the GAL61 strain of *T. cruzi* exposed to continuously increasing drug pressure. A population (61R) was established that grew at a comparable rate in the presence or absence of 50 µM i.e., 13.0 µg/mL BZN. Six clonal lines derived from this population exhibited 3–7-fold increase in $IC_{50}$s (Figure 17A). In the absence of drug, the clones grew slightly slower in culture than the parental cells but otherwise displayed no obvious morphological changes. The 61R cells were cross-resistant to the other nitroheterocycles, nifurtimox (Figure 17B).

[58] Villarreal D, Nirde P, Hide M, Barnabe C, and Tibayrenc M. Differential gene expression in benznidazole-resistant *Trypanosoma cruzi* parasites. *AAC* (2005) 49 (7): 2701-2709.

Figure 17: Properties of *T. cruzi* clones derived by BZN selection. A median inhibitory concentration ($IC_{50}$) of BZN


Campos *et al*., 2013[59] reported the development of resistance *in vitro* to BZN using the epimastigotes of the Y strain of *T. cruzi*. Briefly, $5x10^6$ epimastigotes were cultured with 182.1 µM i.e., 47.4 µg/mL (represent $IC_{50}$ value) of BZN for 24 hours, and incubated in drug-free medium for 7 days. This was followed by 10 cycles of such passages. The drug concentration was increased in 10 µM steps between 11 and 15 passages. The $IC_{50}$ values were measured after each passage. The persistence of the resistant phenotypes was evaluated by cultivating the parasites *in vitro* without drug pressure for 6 months. The results show that the BZN $IC_{50}$ values increased 4.7-fold (from 182.1 to 863.3 µM i.e., 47.4 to 224.7 µg/mL) by the $15^{th}$ passage (Table 10). The resistance induced in the epimastigotes was maintained (the $IC_{50}$ values were increased) after transformation to metacyclic trypomastigotes, cell-derived trypomastigotes, and intracellular amastigotes of the B15 lines (Table 10). Such an effect was irreversible.

[59] Campos MC, Castro-Pinto DB, Ribeiro GA, Berredo-Pinho MM, Gomes LHF, da Silva Bellieny MS, Goular CM, Echevarria A, and Leon LL. P-glycoprotein efflux pump plays an important role in *Trypanosoma cruzi* drug resistance. *Parasitol Res* (2013) 112: 2341–2351.

Table 10: Induction of resistance to BZN in *T. cruzi* epimastigotes and persistence of drug resistant phenotype after transformation to trypomastigotes and amastigotes.

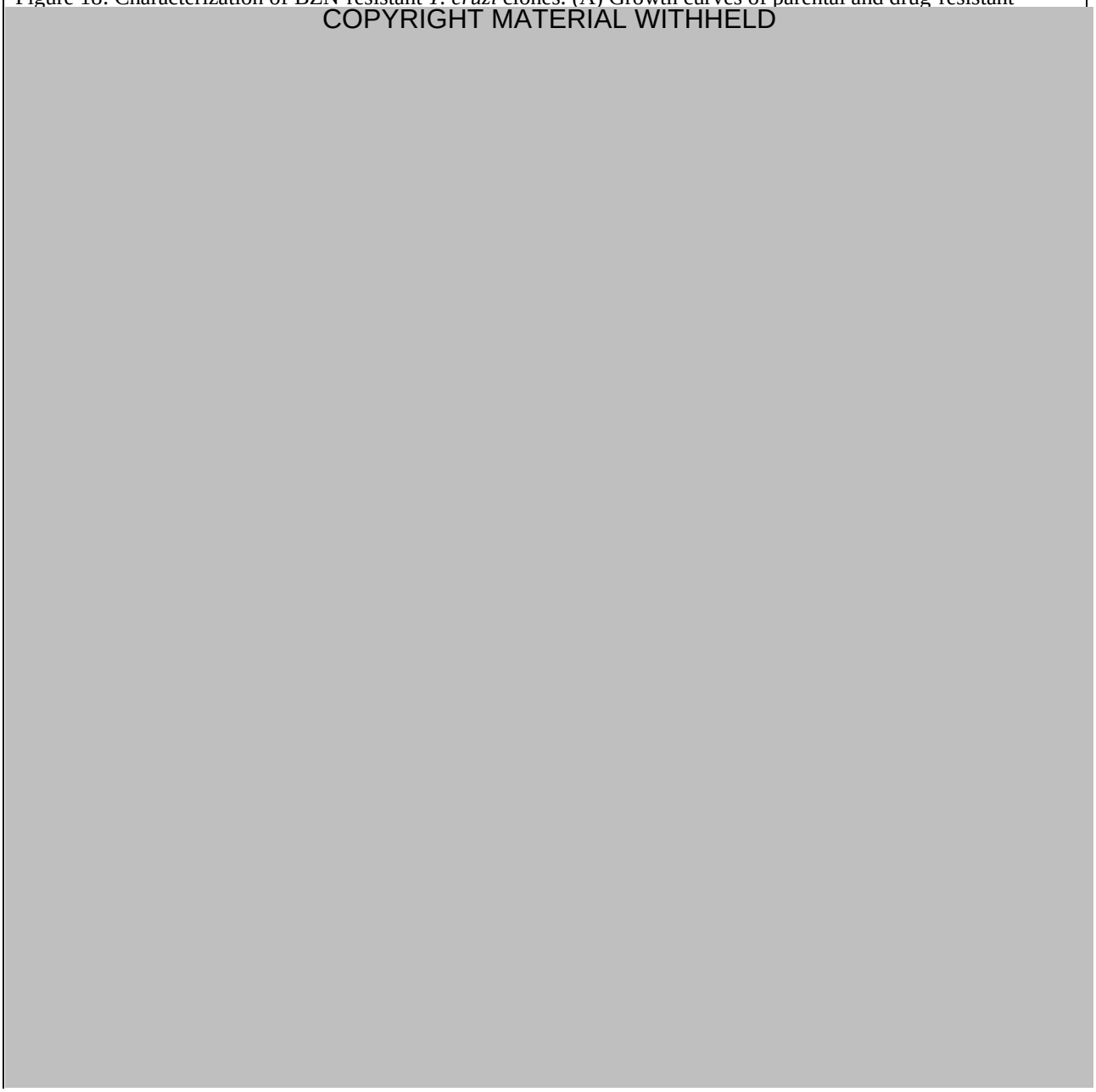


Campos *et al*., 2014[60] evaluated the growth rate of epimastigotes of the three clones of the Y strain of *T. cruzi* derived from a single population which had been selected for resistance by exposure to increasing concentrations of BZN grown in the absence of the drug. The drug-resistant clones grew at about half the rate of the parental non-resistant cells, which had a doubling time of 33 hours. There were significant differences in the level of BZN $IC_{50}$ values among the three clones, ranging from 9 to 26-fold (Figure 18). Each clone was cross-resistant to nifurtimox, at levels ranging from 2 to 4-fold.

There were no significant differences in the rate of amastigote replication or the extent of infectivity between the parental parasites and any of the drug-resistant clones.

[60] Campos MCO, Leon LL, Taylor MC, and Kelly JM. Benznidazole-resistance in *Trypanosoma cruzi*: Evidence that distinct mechanisms can act in concert. *Mol and Biochem Parasitol* (2014) 193: 17-19.

Figure 18: Characterization of BZN-resistant *T. cruzi* clones. (A) Growth curves of parental and drug-resistant




### 3.4.1.2. Mechanism of drug resistance

#### *3.4.1.2.1. Role of efflux pump (P-glycoprotein and ATP-binding casette transporter)*

***P-glycoprotein*:** Campos *et al*., 2013[59] reported an association between p-glycoprotein (Pgp) activity and *T. cruzi* parasites resistant to BZN (after 15 passages *in vitro*) by a Rhodamine 123 fluorescence assay, a molecular probe that mimics some Pgp substrates. The results showed about 50% lower fluorescence within the resistant parasites compared to the parenteral line suggesting increased efflux activity; the BZN $EC_{50}$ values were about 4.7-fold higher in resistant parasites compared to parental parasite. Such an effect was reversed by Pgp inhibitors, cyclosporine A or verapamil (Figure 19).

Figure 19: Histograms showing the $EC_{50}$ values for the treatment of the parental and B15 lines with BZN plus 2 μM of Pgp inhibitors (cyclosporine A or verapamil) measured by the uptake of rhodamine 123 fluorescence in *T. cruzi* epimastigotes.



In *T. cruzi*, the Pgp is encoded by two genes, $TcPGP_1$ and $TcPGP_2$. The expression of both $TcPGP_1$ and $TcPGP_2$ genes was 1.7-fold and 1.6-fold higher in the BZN resistant line compared to the parental line.

***ATP-binding casette transporter*:** Zingales *et al* (2015)[61] and Franco *et al* (2015)[37] reported up-regulation of the expression of the ATP-binding cassette (ABC) transporter gene, *TcABCG1*, gene as well as the protein in the epimastigotes of the resistant strains compared to the susceptible strains of *T. cruzi* (Figure 20). ABC transporters are membrane proteins that mediate the ATP-driven unidirectional transport of a variety of molecules across biological membranes.

Figure 20: Relative transcript abundance of *TcABCG1* transporter in epimastigote forms of BZN-susceptible and BZN-resistant strains.



### *3.4.1.2.2. Role of nitroreductase gene*

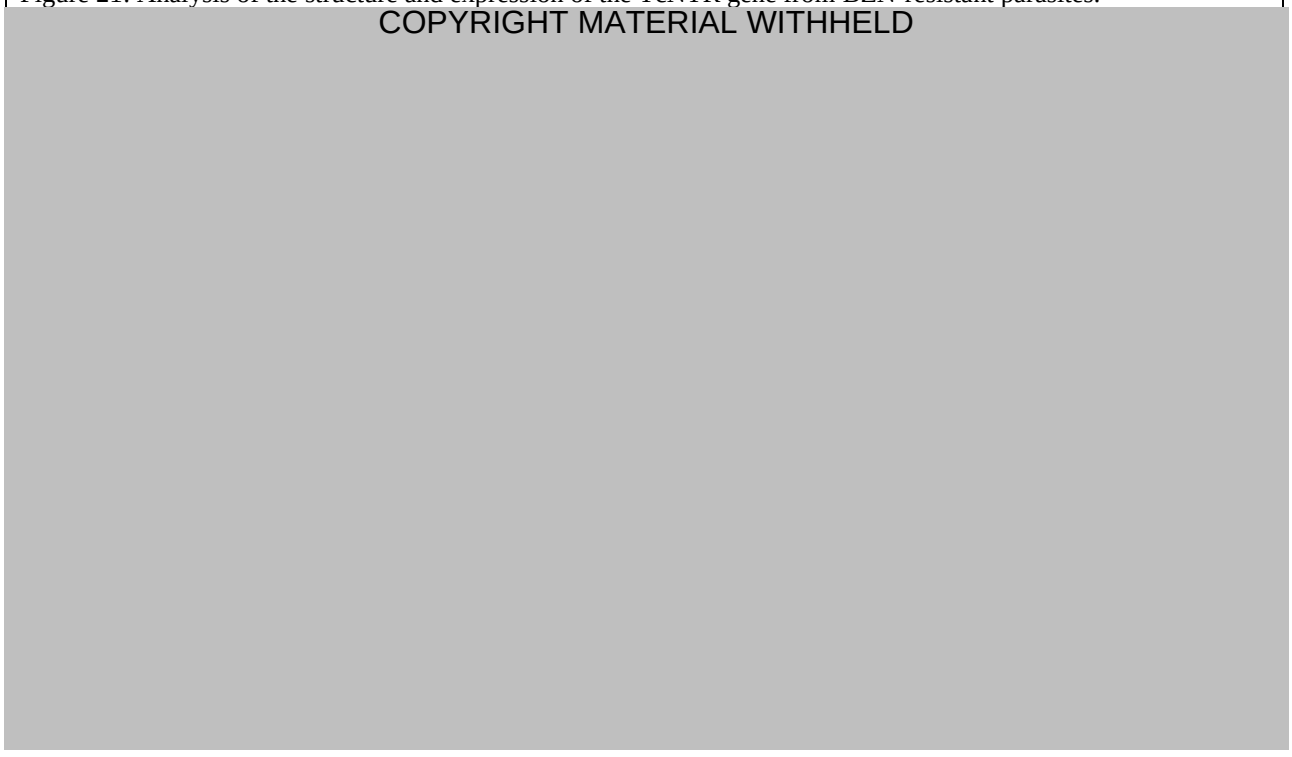
Mejia *et al*., 2012[36] reported the loss of type I *NTR* gene (*TcNTR*) in BZN resistant clone generated *in vitro* compared to the parental cells (for details see section 3.4.1.1. above). Following selection of BZN-resistant parasites, all clones examined had lost one of the chromosomes containing the *TcNTR* gene. In the sensitive parental cells (61S), *TcNTR* was a single copy gene located on chromosome homologues of 1.1-Mb and 0.85-Mb. In the resistant parasites, however, the 0.85-Mb band was missing in clonal and polyclonal populations (Figure 17C, lanes 2 and 3). There were no other apparent changes to the chromosome profile.

[61] Zingales B, Araujo RG, Moreno M, Franco J, Aguiar PHN, Nunes SL, Silva MN, Ienne S, Machado CR, and Brandao A. A novel ABCG-like transporter of *Trypanosoma cruzi* is involved in natural resistance to benznidazole. *Mem Inst Oswaldo Cruz* (2015)110(3):433-444.

To examine that the drug resistance was associated with loss of *TcNTR* rather than another gene located elsewhere on the missing chromosome, an active copy of *TcNTR* was reintroduced by transfection with WT *TcNTR* into 61R clone 2; BZN sensitivity was restored (Figure 17D). However, loss of 1 copy of *TcNTR* did not reduce infectivity. In culture, resistant epimastigotes differentiated into metacyclic trypomastigotes at a level similar to sensitive clones. When culture derived trypomastigotes were used to initiate infections, all the resistant clones tested (clones 3, 4, and 6) were able to develop through the intracellular cycle as amastigotes and differentiate into bloodstream trypomastigotes, which were released following host cell lysis. However, a reduction in virulence was observed at 2 levels; when Vero cells were used, the number of cells infected by resistant clones was significantly less than the level observed with the parental sensitive parasites, and the average number of amastigotes per infected cell was reduced. The authors state that when L6 cells were infected with drug-resistant metacyclics, although released trypomastigotes could be observed, their numbers were too few for a quantifiable infection assay to be performed.

Campos *et al*., 2014[60] examined *T. cruzi* nitroreductase (*TcNTR*) gene, responsible for activating BZN, in the epimastigotes of the three clones of the Y strain of *T. cruzi* derived from a single population which had been selected for resistance by exposure to increasing concentrations of BZN (for details see section 3.4.1.1. above). The results showed a C/T transition at position 568 in each of the clone; this transition generates a stop codon in the middle of the gene leading to a truncated protein deficient in the putative carboxyl terminal FMN-binding site. By PCR, clones 1 and 2 were shown to be homozygous, whereas clone 3 had retained a copy of the non-mutated parental allele (Figure 21). By Northern blots, the level of *TcNTR* RNA expression was increased in all three clones (Figure 21). Each clone had acquired a stop-codon-generating mutation in the gene which encodes the nitroreductase (*TcNTR*). In addition, one clone had lost a copy of the chromosome containing *TcNTR*.

Figure 21: Analysis of the structure and expression of the TcNTR gene from BZN-resistant parasites.



***3.4.1.2.3. Differential gene expression***

Villarreal *et al*., 2005[58] reported no link between drug resistance and genetic clustering by multilocus enzyme electrophoresis (MLEE) or random amplified polymorphic DNA (RAPD) analysis. A total of 19 different isoenzymatic systems corresponding to 22 polymorphic loci were scored. However, variability in the differential expression of gene from resistant parasites belonging to the same genetic cluster, compared to the WT, was observed by the random differentially expressed sequences (RADES) technique (Figure 22).

Figure 22: RADES profiles obtained with primers A7 (a), A9 (b), and U16 (c) for wild-type (WT) and resistant (R)



The study suggests variability in the differential gene expression of resistant parasites belonging to the same genetic cluster. Also, the random distribution of differential bands suggests that each stock acts independently of its own genetic cluster when it is submitted to drug pressure.

Rêgo *et al* (2015)[62] reported twofold higher levels of a cyclophilin *TcCyP19* expression (measured by mRNA), in drug resistant clones (17 LER; obtained *in vitro* by exposure to increasing concentrations of BZN) than the WT (Tehuantepec cl2) strain of *T. cruzi*. This was associated with increased expression of TcCyP19 protein. It was noted that this observation is based on a clone generated *in vitro* as no differences in TcCyP19 mRNA and protein expression levels were observed between the susceptible and the naturally resistant *T. cruzi* strains analyzed. Cyclophilin is known to be important in protein folding, protein–protein interaction cellular stress response, and signal transduction.

[62] Rêgo JV, Duarte AP, Liarte DB, de Carvalho Sousa F, Barreto HM, Bua J, Romanha AJ, Radis-Baptista G, and Murta SMF. Molecular characterization of Cyclophilin (TcCyP19) in *Trypanosoma cruzi* populations susceptible and resistant to benznidazole. *Exp Parasitol* (2015)148: 73-80.

Nogueira *et al* (2006)[63] reported increased expression of the iron-containing superoxide dismutase-A (*TcFeSOD-A*) mRNA in BZN resistant clones compared to the BZN sensitive clone/strains. The increased expression of *TcFeSOD-A* was associated with increased activity of superoxide dismutase (SOD) enzyme. SOD enzyme is an anti-oxidant that removes excess superoxide radicals by converting them to oxygen ($O_2$) and hydrogen peroxide ($H_2O_2$) and is considered to be important for survival.

Rajão *et al* (2014)[30] reported that both overexpression and reduced expression of proteins involved in DNA metabolism alter *T. cruzi* resistance to BZN.

### 3.4.2. Drug resistance *in vivo*

Studies (Marretto *et al*., 1999[64]; Murta and Romanha, 1998[65]) showed resistance of *T. cruzi* strains to BZN and nifurtimox increased when parasites were isolated from mice previously treated with the same drug. The authors suggest that the initial treatment eliminated the sensitive parasites, preserving the resistant ones, which multiplied and dominated the population. This can result in the selection of a subset of the population.

dos Santos *et al*., 2008[66] induced BZN resistance by a continuous drug pressure in mice infected with BE-62 and BE-78 strains (Type II) of *T. cruzi* originally isolated from the patient Berenice in 1962 and 1978, respectively. Two *T. cruzi* stocks were obtained from different outbred dogs chronically infected with BE-62 (BE-62A and BE-62B) 10 years ago, and three were isolated from different dogs infected with the BE-78 strain infected 2 (BE-78D), 7 (BE-78C) and 10 (BE-78E) years ago. The resistant parasites were analyzed to verify stability of resistance during its life cycle *in vivo* and *in vitro*, as well as the effect of BZN on the parasitemia levels evaluated. Mice inoculated with BE-62A and BE-62B strains were 50% and 60% resistant to BZN; mice inoculated with BE-78C, BE-78D and BE-78E stocks were 90%, 70% and 90%, respectively, resistant to the drug. The authors state that "contrary to the other stocks, BE-78D and BE-78E stocks changed to a drug sensitive phenotype after maintenance in mice for two years by successive blood passages."

Swiss outbred mice were inoculated intra-peritoneally with 5000 trypomastigotes of each of the *T. cruzi* strains. Treatment with BZN (100 mg/kg orally for 20 days) was initiated after detection of parasitemia in fresh blood, approximately 10 days post-inoculation. Resistance to BZN was induced by successive cycles of treatment with BZN. Mice that did not show parasitemia reactivation after treatment were immunosuppressed with cyclophosphamide (three cycles of 50 mg of cyclophosphamide/kg, for four consecutive days, with an interval of 3 days between each cycle). Parasitemia was evaluated during and until 5 days after cyclophosphamide treatment. Parasites obtained after immunosuppression were inoculated in to a new group of 10 mice.

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[63] Nogueira FB, Krieger MA, Nirdé P, Goldenberg S, Romanha AJ, and Murta SM. Increased expression of iron-containing superoxide dismutase-A (TcFeSOD-A) enzyme in *Trypanosoma cruzi* population with in vitro-induced resistance to benznidazole. *Acta Trop* (2006) 100(1-2):119-132.

[64] Marretto JPM, Andrade SG 1994. Biochemical behavior of *Trypanosoma cruzi* strains isolated from mice submitted to specific chemotherapy. *Rev Soc Bras Med Trop 27*: 209-215.

[65] Murta SMF and Romanha AJ. *In vivo* selection of a population of *Trypanosoma cruzi* and clones resistant to BZN. *Parasitology* (1998) 116: 165-171.

[66] dos Santos FM, Caldos S, de Assis Cau SB, Crepalde GP, de Lana M, Machado-Coelho GLL, Veloso VM, and Bahia MT. *Trypanosoma cruzi*: Induction of benznidazole resistance *in vivo* and its modulation by in vitro culturing and mice infection. *Experimental Parasitology* (2008) 120: 385–390.

Successive treatment cycles (TC) were performed until fully BZN-resistant parasites were obtained for at least four successive TC. To verify the stability of the phenotype, parasites isolated from the last TC were maintained by successive blood passages in untreated mice or in acellular culture medium for six months. After this, *T. cruzi* resistant stocks were inoculated into a new group of animals and both drug resistance and biological properties (parasitemia, mortality and virulence) evaluated.

Parasitological cure was assessed by three independent tests, before, during and after cyclophosphamide immunosuppression, by fresh blood examination, blood culture and PCR. Animals were considered cured when all tests were persistently negative.

BZN-resistant parasites were obtained from all *T. cruzi* stocks except BE-62A submitted to the long-term BZN-pressure protocol; BE-62A showed 50% resistance to BZN in the first TC, by the PCR assay only. The other *T. cruzi* stocks evaluated were resistant after 2–9 successive cycles of BZN-treatment. However, the number of TC necessary to obtain fully resistant parasites as well as the time (4–18 months) necessary to induce resistance varied among the different *T. cruzi* stocks (Figure 23). During the $10^{th}$ TC, BZN was not effective in suppression of parasitemia even during the treatment period (Figure 24).

Figure 23: Cure percentage during successive cycles of BZN treatment in mice inoculated with BE-62B (A), BE-

Figure 24: Parasitemia curve of BE-78C stock in mice treated



Stability of the resistant phenotype *in vitro* and *in vivo* was measured using the stock BE-78C. The resistance phenotype remained stable after *T. cruzi* stocks were maintained by 12 passages in mice for six months and in acellular culture for the same time (Table 11). The maintenance of resistant parasite for 12 months in acellular culture induced a reduction in the level of BZN resistance; however, no alteration was detected in parasites maintained for the same time in mice (Table 11). The study suggests the stability of resistance acquired under drug pressure *in vivo*; however, there is a possibility of reversible changes in the resistance levels after maintenance for long time in acellular culture *in vitro* but not *in vivo*.

Table 11: Determination of the stability of BZN resistance after passage of resistant parasites in mice and



The different stocks, before and after the first and the last TC with BZN, were inoculated in to experimentally naïve mice to determine parasitemia levels. The results showed differences in parasitemia levels and time to patency for some of the stocks collected before and after treatment cycles (Figure 25). The authors state that there was no difference in infectivity.

Figure 25: Parasitemia curves of *T. cruzi* stocks in mice inoculated with blood trypomastigotes obtained from mice


Filardi and Brener (1987)[67] reported the activity of BZN against 51 strains of *T. cruzi* (frozen in liquid nitrogen in the cryobank, WHO Collaborating Centre for the Cryopreservation of American Trypanosoma - maintained at the Laboratory of Chagas Disease in the Centro de Pesquisas Rene Rachouin in Belo Horizonte). Of the 47 strains, 26 were isolated from humans, 7 from the strictly intra-domiciliary vector *Triatoma infestans*, 8 from the sylvatic vector *Panstrongylus megistus*, 1 from a domestic cat and 5 from wild reservoirs. Albino mice were infected with $10^4$ *T. cruzi* blood forms and treated orally from Day 4 PI with 100 mg/kg BZN for 20 consecutive days. Blood was examined microscopically for living flagellates starting 10 days after treatment and performed 2-5 times over a period of 15 days. Hemoculture was performed 30 to 45 days after the end of treatment on treated and surviving mice. IFA was performed using formalin-fixed amastigotes and trypomastigotes grown in Vero cell cultures. The results showed that the cure rates varied from 0 to 100% (Table 12). There appears to be a higher sensitivity of the southern strains (Figure 26C) compared to other strains (Figure 26 B and D). Strains from animal reservoirs also showed a wide range of drug sensitivity (Table 12 and Figure 26). The authors used an arbitrary level of 50% cure as a criterion for discriminating naturally drug-resistant and drug-sensitive *T. cruzi* population;13 strains (27.6%) were considered as resistant to BZN. It is unclear if the cure rates were based on hemoculture as well as IFA.

[67] Filardi LS and Brener Z. Susceptibility and natural resistance *Trypanosoma cruzi* strains to drugs used clinically in Chagas disease. *Trans Roy Soc Trop Med Hyg* (1987) 81: 755-759.

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Figure 26: Percentage of cure in groups of mice inoculated with different *T. cruzi* strains and treated with nifurtimox

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*Comments:*
*Studies in vitro as well as in murine models of T. cruzi suggest a potential for development of resistance to BZN. The in vitro $IC_{50}$ values were higher under increasing drug pressure. The resistant clones/strains grow slower than the parenteral strain in vitro. There is no effect on the rate of amastigote replication or the extent of infectivity between the parental parasites and any of the drug-resistant clones. The viability of the parasites remained unaltered. Similarly, the*

*cure rates were lower in mice infected with the resistant strains/clones. The initial treatment eliminated the sensitive parasites, preserving the resistant ones, which multiplied and dominated the population. Thus natural and artificial pressure can result in the selection of a subset of the population. A study suggest that BZN resistance may be reversed after T. cruzi stocks are kept for a long time in acellular culture in a drug free medium in vitro. However, such an effect was not observed in vivo.*

*The mechanism of resistance may be multifactorial; studies suggest higher efflux activity of Pgp, increased expression of both $TcPGP_1$ and $TcPGP_2$ genes as well as mutation or different gene expression the T. cruzi nitroreductase (TcNTR) gene to be associated with resistance to BZN.*

## 4. CLINICAL MICROBIOLOGY

The Applicant submitted several published studies to support the efficacy and safety of BZN in children (b) (4) with Chagas disease. The datasets were included for 4 studies (Sosa-Estani *et al*., 1998[1]; de Andrade *et al*., 1996[2]; Molina *et al*., 2014[3]; DNDi-CH-E1224-001); these 4 studies as well as some of the supportive information from published studies are summarized below.

The serological and/or RT-PCR assays were used for patient enrollment as well as measuring efficacy. In general, the details of the methods and performance characteristics of the assays used supporting the use of these tests for the intended context of use i.e., enrollment of subjects and measuring efficacy were not included in the publications; the authors cross-referenced other publications for the methods used. The Applicant provided the publications that were cross-referenced in the published studies as well as a Laboratory standardization manual[68]; the latter included a brief synopsis of the methods used. The Applicant states that the validation of the assays performed does not meet the current scientific and regulatory standards. The CDRH was consulted for the appropriateness of the serological assays used in the clinical studies for the intended context of use.

### 4.1. Study 1 (Sosa-Estani *et al*., 1998)

This was a randomized, double blind, placebo controlled trial to evaluate the efficacy and tolerance of treatment with BZN (Radanil®; Roche, Olivios, Argentina) in 106 children, 6-12 years of age, infected with *T. cruzi* in the indeterminate phase of Chagas disease (Sosa-Estani *et al*., 1998[1]). The study was conducted between 1991 and 1995.

**Study design**

The children were from 14 localities in the Province of Salta in North-Western Argentina, where prevention methods for the reduction of triatomines have been continuously used since 1982. Therefore, it was assumed that the infection of these children must be longer than 6 years ago. Children were excluded from the trial for any of the following reasons:

- Presence of any chronic health condition (such as epilepsy, malnutrition, asthma, severe anemia).
- Presence of any acute infectious disease.
- Lack of consent from their parents to participate in the study.
- Unstable residence.

[68] Chemo Research, SL. Laboratory standardization manual: Serological and molecular testing of efficacy endpoints in clinical studies. December 16, 2016 (version 1.3; final version).

Treatment with BZN (5 mg/kg/day) or placebo was administered for 60 days and subjects were followed for 48 months for clinical and serological response at different time intervals. Clinical examination included continuous physical examinations, anamnesis, and ECGs. Serological assessments were performed immediately before and at 3, 6, 12, 18, 24 and 48 months after the onset of treatment by 4 different tests: IHA, IFA, conventional ELISA, and non-conventional ELISA (F29-ELISA). None of the assays used are FDA cleared tests.

All serological testing was performed at (b) (4) (b) (4). The authors[1] cross-referenced other publications (Cerisola *et al*., 1971[69] and 1974[70]; Alvarez *et al*., 1968[71]; Voller *et al*., 1975[72]; Porcel *et al*., 1996[73]; Engman *et al*., 1989[74]) for the methods. The Applicant has included the cross-referenced publications as well as brief details of the methods used for serological and parasitological testing in the Laboratory standardization manual.[68] It was noted that the study was published in 1998 and the Lab manual dated 1999; it is possible that the lab manual was modified over a period of time. The differences noted in the information available from the referenced publications compared to that in the Laboratory standardization manual are summarized in Appendix-3, Table A. These differences can alter the performance of the test. There were no data available to assess the day to day variability of the assays, the positive or negative controls (calibrators) included for testing as well as data to support the cut-offs used to characterize a subject as seropositive or seronegative.

Parasitological measurement, by xenodiagnosis, was performed at Months 24 and 48 in some of the patients.

**Results**

There is a lot of variability in antibody titers by all the four serological tests. The results showed a trend towards a decrease in the number of subjects that became seronegative as well as antibody titers in BZN group compared to the placebo group by the four tests (Figure 27 and Table 13). The difference in the percentage of subjects that became seronegative, in the untreated group compared to BZN treated group, was statistically significant by F29-ELISA and not by the other serological tests from Month 12 after the onset of treatment.

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[69] Cerisola JA, Álvarez M, de Martini GJW, and Bonacci HJ. Qualitative haemagglutination assay for diagnosing Chagas disease. *Bioquímica Clínica* (1971) V ( 2): 94-98. The Applicant provided English translation of the publication.

[70] Cerisola JA, Rohweder R, Segura EL, Del Prado CE, Alvarez M, Wynne de Martini GJ. El Xenodiagno´stico. Normatizacion, Utilidad. Buenos Aires: Public Secretarıa de Salud Publica, Ministerio de Bienestar Social. 1974.

[71] Alvarez M, Cerisola JA, and Rohweder RW. Test de inmunofluorescencia para el diagnostico de la enfermedad de Chagas. Bol Chil Parasitol (1968) 23: 4–8. The Applicant provided an English translation of the publication.

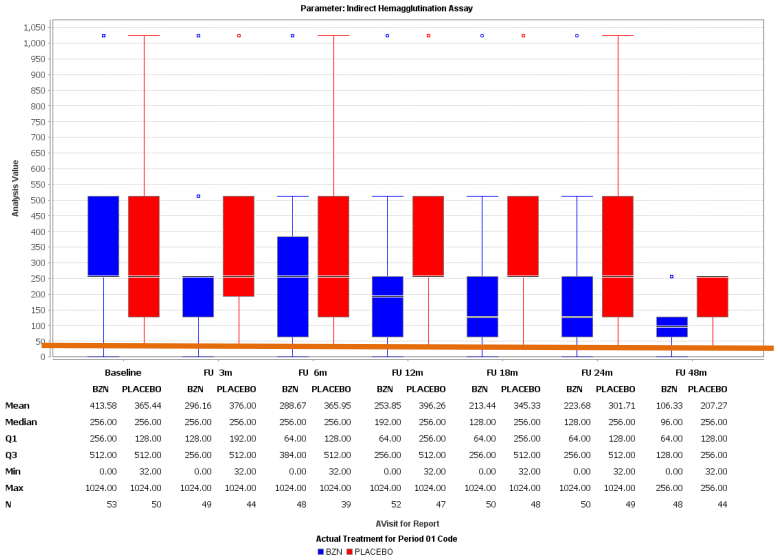
[72] Voller A, Draper C, Bidwell DE, and Bartlett A. Microplate enzyme linked immunosorbent assay for Chagas disease. *The Lancet* (1975) 305 (7904): 426-428.

[73] Porcel B, Bontempi E, Heriksson, Rydaker M, Aslund L, Segura EL, Petterson U, Ruiz AM. *Trypanosoma rangeli* and *Trypanosoma cruzi*: molecular characterization of genes encoding putative calcium-binding proteins, highly conserved in trypanosomatids. *Exp Parasitol* (1996) 84: 387–399.

[74] Engman DM, Kraus K-H, Blumin JH, Kim KS, Kirchhoff LV, and Donelson JE. A Novel Flagellar Ca2+-binding Protein in Trypanosomes. *J Biol Chemistry* (1989) 264 (31): 18627-18631.

Figure 27: Study 1 (Sosa-Estani *et al*., 1998[1]) – Antibody titers (Box whisker plots: Median and % quartile represented by bars) by IHA, IFA, conventional ELISA and F29-ELISA at different time points.

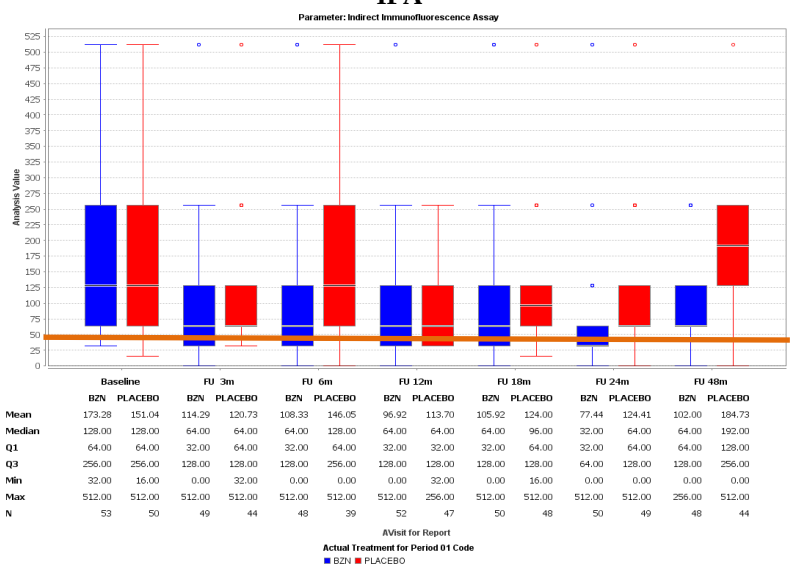
**IHA**




| | Baseline | | FU 3m | | FU 6m | | FU 12m | | FU 18m | | FU 24m | | FU 48m | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BZN | PLACEBO | BZN | PLACEBO | BZN | PLACEBO | BZN | PLACEBO | BZN | PLACEBO | BZN | PLACEBO | BZN | PLACEBO |
| Mean | 413.58 | 365.44 | 296.16 | 376.00 | 288.67 | 365.95 | 253.85 | 396.26 | 213.44 | 345.33 | 223.68 | 301.71 | 106.33 | 207.27 |
| Median | 256.00 | 256.00 | 256.00 | 256.00 | 256.00 | 256.00 | 192.00 | 256.00 | 128.00 | 256.00 | 128.00 | 256.00 | 96.00 | 256.00 |
| Q1 | 256.00 | 128.00 | 128.00 | 192.00 | 64.00 | 128.00 | 64.00 | 256.00 | 64.00 | 256.00 | 64.00 | 128.00 | 64.00 | 128.00 |
| Q3 | 512.00 | 512.00 | 256.00 | 512.00 | 384.00 | 512.00 | 256.00 | 512.00 | 256.00 | 512.00 | 256.00 | 512.00 | 128.00 | 256.00 |
| Min | 0.00 | 32.00 | 0.00 | 32.00 | 0.00 | 32.00 | 0.00 | 32.00 | 0.00 | 32.00 | 0.00 | 32.00 | 0.00 | 32.00 |
| Max | 1024.00 | 1024.00 | 1024.00 | 1024.00 | 1024.00 | 1024.00 | 1024.00 | 1024.00 | 1024.00 | 1024.00 | 1024.00 | 1024.00 | 256.00 | 256.00 |
| N | 53 | 50 | 49 | 44 | 48 | 39 | 52 | 47 | 50 | 48 | 50 | 49 | 48 | 44 |

≥1/32 dilution represent positive

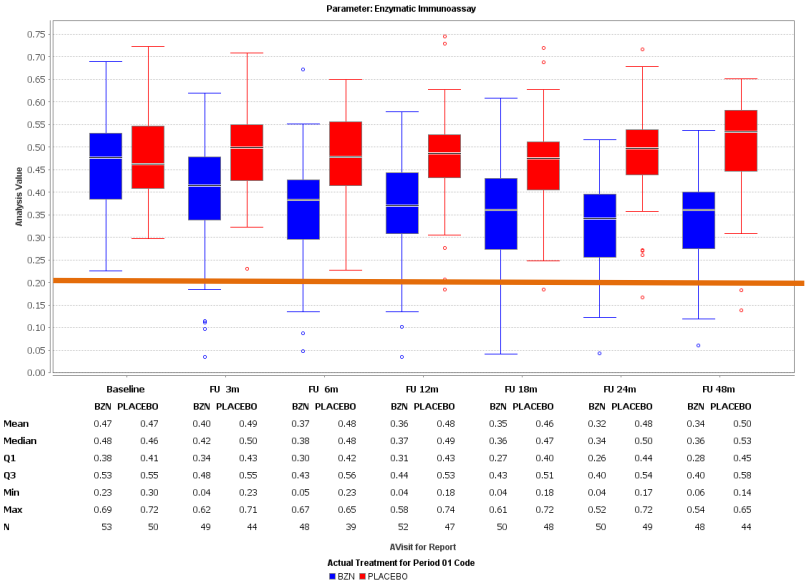
**IFA**




| | Baseline | | FU 3m | | FU 6m | | FU 12m | | FU 18m | | FU 24m | | FU 48m | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BZN | PLACEBO | BZN | PLACEBO | BZN | PLACEBO | BZN | PLACEBO | BZN | PLACEBO | BZN | PLACEBO | BZN | PLACEBO |
| Mean | 173.28 | 151.04 | 114.29 | 120.73 | 108.33 | 146.05 | 96.92 | 113.70 | 105.92 | 124.00 | 77.44 | 124.41 | 102.00 | 184.73 |
| Median | 128.00 | 128.00 | 64.00 | 64.00 | 64.00 | 128.00 | 64.00 | 64.00 | 64.00 | 96.00 | 32.00 | 64.00 | 64.00 | 192.00 |
| Q1 | 64.00 | 64.00 | 32.00 | 64.00 | 32.00 | 64.00 | 32.00 | 32.00 | 32.00 | 64.00 | 32.00 | 64.00 | 64.00 | 128.00 |
| Q3 | 256.00 | 256.00 | 128.00 | 128.00 | 128.00 | 256.00 | 128.00 | 128.00 | 128.00 | 128.00 | 64.00 | 128.00 | 128.00 | 256.00 |
| Min | 32.00 | 16.00 | 0.00 | 32.00 | 0.00 | 0.00 | 0.00 | 32.00 | 0.00 | 16.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Max | 512.00 | 512.00 | 512.00 | 512.00 | 512.00 | 512.00 | 512.00 | 1024.00 | 512.00 | 512.00 | 512.00 | 512.00 | 256.00 | 512.00 |
| N | 53 | 50 | 49 | 44 | 48 | 39 | 52 | 47 | 50 | 48 | 50 | 49 | 48 | 44 |

≥1/32 dilution represent positive

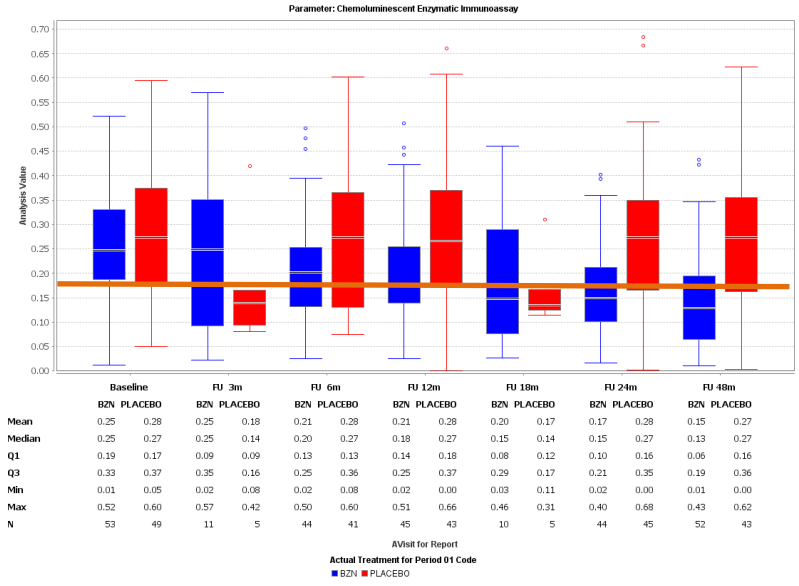
**Conventional ELISA**




| | Baseline | | FU 3m | | FU 6m | | FU 12m | | FU 18m | | FU 24m | | FU 48m | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BZN | PLACEBO | BZN | PLACEBO | BZN | PLACEBO | BZN | PLACEBO | BZN | PLACEBO | BZN | PLACEBO | BZN | PLACEBO |
| Mean | 0.47 | 0.47 | 0.40 | 0.49 | 0.37 | 0.48 | 0.36 | 0.48 | 0.35 | 0.46 | 0.32 | 0.48 | 0.34 | 0.50 |
| Median | 0.48 | 0.46 | 0.42 | 0.50 | 0.38 | 0.48 | 0.37 | 0.49 | 0.36 | 0.47 | 0.34 | 0.50 | 0.36 | 0.53 |
| Q1 | 0.38 | 0.41 | 0.34 | 0.43 | 0.30 | 0.42 | 0.31 | 0.43 | 0.27 | 0.40 | 0.26 | 0.44 | 0.28 | 0.45 |
| Q3 | 0.53 | 0.55 | 0.48 | 0.55 | 0.43 | 0.56 | 0.44 | 0.53 | 0.43 | 0.51 | 0.40 | 0.54 | 0.40 | 0.58 |
| Min | 0.23 | 0.30 | 0.04 | 0.23 | 0.05 | 0.23 | 0.04 | 0.18 | 0.04 | 0.18 | 0.04 | 0.17 | 0.06 | 0.14 |
| Max | 0.69 | 0.72 | 0.62 | 0.71 | 0.67 | 0.65 | 0.58 | 0.74 | 0.61 | 0.72 | 0.52 | 0.72 | 0.54 | 0.65 |
| N | 53 | 50 | 49 | 44 | 48 | 39 | 52 | 47 | 50 | 48 | 50 | 49 | 48 | 44 |

Cut-off OD >0.200

**Non-conventional F29-ELISA**




| | Baseline | | FU 3m | | FU 6m | | FU 12m | | FU 18m | | FU 24m | | FU 48m | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BZN | PLACEBO | BZN | PLACEBO | BZN | PLACEBO | BZN | PLACEBO | BZN | PLACEBO | BZN | PLACEBO | BZN | PLACEBO |
| Mean | 0.25 | 0.28 | 0.25 | 0.18 | 0.21 | 0.28 | 0.21 | 0.28 | 0.20 | 0.17 | 0.17 | 0.28 | 0.15 | 0.27 |
| Median | 0.25 | 0.27 | 0.25 | 0.14 | 0.20 | 0.27 | 0.18 | 0.27 | 0.15 | 0.14 | 0.15 | 0.27 | 0.13 | 0.27 |
| Q1 | 0.19 | 0.17 | 0.09 | 0.09 | 0.13 | 0.13 | 0.14 | 0.18 | 0.08 | 0.12 | 0.10 | 0.16 | 0.06 | 0.16 |
| Q3 | 0.33 | 0.37 | 0.35 | 0.16 | 0.25 | 0.36 | 0.25 | 0.37 | 0.29 | 0.17 | 0.21 | 0.35 | 0.19 | 0.36 |
| Min | 0.01 | 0.05 | 0.02 | 0.08 | 0.02 | 0.08 | 0.02 | 0.00 | 0.03 | 0.11 | 0.02 | 0.00 | 0.01 | 0.00 |
| Max | 0.52 | 0.60 | 0.57 | 0.42 | 0.50 | 0.60 | 0.51 | 0.66 | 0.46 | 0.31 | 0.40 | 0.68 | 0.43 | 0.62 |
| N | 53 | 49 | 11 | 5 | 44 | 41 | 45 | 43 | 10 | 5 | 44 | 45 | 52 | 43 |

Cut-off OD≥0.170

FU=follow-up; EOT=end of treatment; ■ Placebo; ■ Benznidazole

Table 13: Study 1 (Sosa-Estani *et al*., 1998[1]) - Number (%) seronegative or parasitologically negative* subjects by different serological tests at baseline and follow-up at Months 3, 6, 12, 24 and 48.

| Test | Treatment arm | Visit | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Baseline | Month 3 | Month 6 | Month 12 | Month 18 | Month 24 | Month 48 |
| **IHA** | Placebo (n=51) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 ( 0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| | Benznidazole (n=55) | 1 (1.8) | 6 (10.9) | 5 (9.1) | 5 (9.1) | 7 (12.7) | 5 (9.1) | 6 (10.9) |
| **IFA** | Placebo (n=51) | 2 (3.9) | 0 (0.0) | 1 (2.0) | 0 (0.0) | 2 (3.9) | 4 (7.8) | 1 (2.0) |
| | Benznidazole (n=55) | 0 (0.0) | 5 (9.1) | 3 (5.5) | 7 (12.7) | 6 (10.9) | 9 (16.4) | 3 (5.5) |
| **Conventional ELISA** | Placebo (n=51) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (2.0) | 1 (2.0) | 1 (2.0) | 2 (3.9) |
| | Benznidazole (n=55) | 0 (0.0) | 5 (9.1) | 4 (7.3) | 5 (9.1) | 5 (9.1) | 7 (12.7) | 4 (7.3) |
| **F29-ELISA** | Placebo (n=51) | 12 (23.5) | 4 (7.8) | 12 (23.5) | 10 (19.6) | 4 (7.8) | 12 (23.5) | 13 (25.5) |
| | Benznidazole (n=55) | 13 (23.6) | 4 (7.3) | 17 (30.9) | 21 (38.2) | 6 (10.9) | 26 (47.3) | 35 (63.6) |
| **Xenodiagnosis*** | Placebo (n=51) | ND | ND | ND | ND | ND | 13 (25.5) | 9 (17.6) |
| | Benznidazole (n=55) | ND | ND | ND | ND | ND | 23 (41.8) | 20 (36.4) |

Patients with a missing value were imputed as seropositive
*Xenodiagnosis performed at Months 24 and 48
EOT=end of treatment; ND=not done

Xenodiagnosis was performed at Months 24 and 48 in some of the patients. A majority (~95%) of the subjects in the BZN group were negative by xenodiagnosis whereas all patients in the placebo group were positive. There was good concordance between the results of the serological tests and positive findings by xenodiagnosis at both Months 24 and 48 (Table 14).

Table 14: Study 1 (Sosa-Estani *et al*., 1998[1]) - Comparison of the results by xenodiagnosis positive subjects with the serological tests at Months 24 and 48

| Positive by serological tests | Positive by xenodiagnosis at Month 24 15/51 (29.4%) | | | | Positive by xenodiagnosis at Month 48 10/39 (25.6%) | | | |
|---|---|---|---|---|---|---|---|---|
| | Placebo [14/27 (51.9%)] | | Benznidazole [1/24 (4.2%)] | | Placebo [9/18 (50.0%)] | | Benznidazole [1/21 (4.8%)] | |
| | Positive | Negative | Positive | Negative | Positive | Negative | Positive | Negative |
| At Month 24 | | | | | | | | |
| IHA | 14/14 | 0/14 | 1/1 | 0/1 | 9/9** | 0/9** | 1/1** | 0/1** |
| IFA | 14/14 | 0/14 | 1/1 | 0/1 | 9/9** | 0/9** | 1/1** | 0/1** |
| ELISA[!] | 14/14 | 0/14 | 1/1 | 0/1 | 9/9** | 0/9** | 1/1** | 0/1** |
| F29-ELISA | 13/13 | 0/13 | 1/1 | 0/1 | 6/7** | 1/7** | ND | ND |
| At Month 48 | | | | | | | | |
| IHA | 13/13* | 0/13* | 1/1* | 0/1* | 9/9 | 0/9 | 1/1 | 0/1 |
| IFA | 13/13* | 0/13* | 1/1* | 0/1* | 9/9 | 0/9 | 1/1 | 0/1 |
| EIA | 13/13* | 0/13* | 1/1* | 0/1* | 9/9 | 0/9 | 1/1 | 0/1 |
| F29-ELISA | 11/13* | 2/13* | 1/1* | 0/1* | 6/7 | 1/7 | 1/1 | 0/1 |

Analysis based on the number of patients tested.
*Xenodiagnosis not done at 48 hours; **Xenodiagnosis not done at 24 hours
[!]Conventional ELISA

There is less concordance between the results by different serological tests and negative findings by xenodiagnosis (Table 15). Majority of the subjects were that were negative by xenodiagnosis were seropositive by the conventional serological tests (IHA, IFA, and ELISA) in both the placebo and BZN groups (Table 15). By F29-ELISA, approximately 50% of the subjects in the

placebo group were seropositive at Months 24 and 48. In the BZN treated group, about 56% and 74% of the subjects were seropositive at Months 24 and 48, respectively (Table 15). Lower concordance between negative findings by xenodiagnosis and serology could be due to lower sensitivity of the xenodiagnosis assay.

Table 15: Study 1 (Sosa-Estani *et al*., 1998[1]) - Comparison of the results by xenodiagnosis negative subjects with the serological tests at Months 24 and 48

| Positive by serological tests | Negative by xenodiagnosis at Month 24 36/51 (70.6%) | | | | Negative by xenodiagnosis at Month 48 29/39 (%) | | | |
|---|---|---|---|---|---|---|---|---|
| | Placebo 13/27 (48.1%) | | Benznidazole 23/24 (95.8%) | | Placebo 9/18 (50.0%) | | Benznidazole 20/21 (95.2%) | |
| | Positive | Negative | Positive | Negative | Positive | Negative | Positive | Negative |
| At Month 24 | | | | | | | | |
| IHA | 12/12 (100.0) | 0/12 (0.0) | 22/23 (95.7) | 1/22 (4.5) | 9/9** (100.0) | 0/9** (0.0) | 18/20** (90.0) | 2/20** (10.0) |
| IFA | 10/13 (76.9) | 3/13 (23.1) | 19/23 (82.6) | 4/23 (17.4) | 9/9** (100.0) | 0/9** (0.0) | 16/20** (80.0) | 4/20** (20.0) |
| ELISA[!] | 12/13 (92.3) | 1/13 (7.7) | 21/23 (91.3) | 2/23 (8.7) | 9/9** (100.0) | 0/9** (0.0) | 16/20** (80.0) | 4/20** (20.0) |
| F29-ELISA | 5/10 (50.0%) | 5/10 (50.0%) | 8/18 (44.4%) | 10/18 (55.6%) | 5/9** (55.6%) | 4/9** (44.4%) | 5/19** (26.3%) | 14/19** (73.7%) |
| At Month 48 | | | | | | | | |
| IHA | 12/12* (100.0) | 0/12* (0.0) | 20/22* (90.9) | 2/22* (9.1) | 9/9 (100.0) | 0/9 (0.0) | 18/20 (90.0) | 2/20 (10.0) |
| IFA | 12/12* (100.0) | 0/12* (0.0) | 20/22* (90.9) | 2/22* (9.1) | 8/9 (88.9) | 1/9 (11.1) | 19/20 (95.0) | 1/20 (5.0) |
| EIA | 11/12* (91.7) | 1/12* (8.3) | 19/22* (86.4) | 3/22* (9.1) | 8/9 (88.9) | 1/9 (11.1) | 18/20 (90.0) | 2/20 (10.0) |
| F29-ELISA | 8/13* (61.5%) | 5/13* (38.5%) | 7/23* (30.4%) | 16/23* (69.6%) | 4/8 (50.0%) | 4/8 (50.0%) | 5/19 (26.3%) | 14/19 (73.7) |

Analysis based on the number of patients tested.
*Xenodiagnosis not done at 48 hours; ** Xenodiagnosis not done at 24 hours
[!]Conventional ELISA

*Comments*
*The antibody response was evaluated by 4 different serologic tests (IHA, IFA, conventional ELISA, and F29-ELISA). This is in accordance with the CDC and WHO recommendations to use at least 2 or 3 serological tests, respectively, to strengthen the validity of the obtained results for diagnosis of patients with Chagas disease. The authors cross-referenced other publications for the methods. Testing was done in* (b) (4) *laboratory. The Applicant provided brief details of the methods used for serological and parasitological testing as well as the cut-offs that were used for characterizing the test results positive or negative in* (b) (4) *laboratory. It was noted that the study was published in 1998 and the information in the Lab manual is dated 1999; there is possibility that the manual was updated as the methods may have evolved over time. Some differences were noted in the information available from the cross-referenced publications compared to that in the Laboratory standardization manual; such differences can alter the performance of the test. No data were available to support the cut-offs used to characterize a subject as seropositive or seronegative.*

*There is a lot of variability in antibody titers by each of the four serological tests used. Overall, the results show a trend towards a decrease in antibody titers as well as the number of subjects that became seronegative in BZN group compared to the placebo group by the four tests; this is based on the cut-offs specified by the Applicant. However, such differences in the antibody titers*

*or the percentage of subjects that became seronegative were statistically significant by F29-ELISA from Month 12 and not by the other serological tests (for details see statistics review). The differences in antibody response by conventional serological tests compared to the non-conventional F29-ELISA appears to be due to the difference in the antigens used for the conventional assays compared to the non-conventional assays. The whole epimastigote was used for the IFA whereas lysate of the parasites was used as antigen for IHA and conventional ELISA (see Appendix-3, Table A). The F29 antigen is a recombinant antigen and represents a purified antigen which was originally extracted from the flagellum of the epimastigotes of the parasite. The antibody response to F29 antigen may be a reflection of response to viable parasite; this is based on published studies that suggest lytic antibodies, measured by a CoML assay, are directed against the live trypomastigotes. Lytic antibodies did not persist in subjects that were negative by xenodiagnosis.*

*By xenodiagnosis, performed at Months 24 and 48 in some of the patients, a majority (~95%) of the subjects in the BZN group were negative by xenodiagnosis whereas placebo group of patients were positive. There was good concordance between the results of the serological tests and positive findings by xenodiagnosis at Month 24 as well as Month 48. However, there was less concordance between negative findings by xenodiagnosis and the results by different serological tests; this could be due to lower sensitivity of the xenodiagnosis assay.*

*The clinical relevance of the test results was not reported in this study. However, another study published by the same group (Sosa-Estani et al., 2002[5]) reported the results of serological testing (IHA, IFA, F29 and antigen trypomastigote (AT) immunoenzymatic tests (IET) in 252 children living in suburban and rural regions (Salta, Santiago del Estero and Catamarca) in Argentina. Xenodiagnosis was performed at 4 and 9 years post-treatment. Vector control measures were in place in the dwellings in these areas. There were 46 subjects from Salta; it appears that the patients treated with BZN from this region were part of the Study 1(Sosa-Estani et al., 1998[1]; summarized above). The subjects were followed for antibody positivity. The details of the methods used and the laboratory where testing of clinical specimens was performed were not included. The post-treatment follow-up varied between 2 and 9 years. The results show a decline in the number of subjects that were antibody positive, compared to pre-treatment, at Month 48 (4 years) by F29 and AT assays (Table 16). About 4 to 5% of the subjects were positive by xenodiagnosis at 4 and 9 years; this is similar to the observations reported previously by Sosa-Estani et al., 1998[1] (see above). The authors state that no significant changes in the ECGs were observed in this population during follow-up.*

Table 16: Sosa-Estani *et al*., 2002[5]- Serology reactivity rates or positive xenodiagnosis in children infected with *T. cruzi* indeterminate chronic phase, treated with BZN. Long-term follow-up.

**4.2. Study 2 (de Andrade *et al*., 1996)**

de Andrade *et al*., 1996[2] reported the results of a randomized double-blind trial to evaluate the efficacy of BZN (Radanil, Roche, Brazil) in the treatment of early *T. cruzi* infection, conducted between 1991 and 1995, in school children between the age of 7 and 12 years from a rural area (three small communities within the north of Goiás) in central Brazil known to be endemic for Chagas disease. The trial was conducted between 1991 and 1995; blood samples were collected by finger prick, on a filter paper, from 1990 school children. Children were screened for anti-*T. cruzi* antibodies by IHA, IFA, and conventional ELISA. Vector control measures were implemented every 6 months throughout the trial; all dwellings in the study communities were sprayed with deltamethrin. This reduced the risk of reinfection. To be eligible for the trial, children had to be seropositive for antibodies to *T. cruzi* by three tests, IHA, IFA, and ELISA.

The primary efficacy endpoint was the disappearance of specific antibodies (negative seroconversion) by the end of 3-year follow-up. The secondary endpoint was ≥3-fold reduction of antibody titers on repeated serological tests.

***Study design***

Of the 1990 children screened, 130 subjects were seropositive in all tests and were randomly assigned to BZN (7.5 mg/kg daily for 60 days by mouth) or placebo groups. Clinical examination, ECG, and laboratory tests were done for all 130 potential trial participants. Clinical examination was performed at some of follow-up visits. All children were symptom free. One child in the BZN group moved away from the area just after randomization and was excluded from analysis. All subjects were tested by IHA, IFA, conventional ELISA and AT-chemiluminescent (CL)-ELISA (non-conventional ELISA) at different time points that include baseline, Day 60 (at the end of treatment), and at Months 3, 6, 12, and 36 after completion of treatment. However, the data were included for Month 36 only.

None of the serological assays used are FDA cleared tests. IHA, IFA and conventional ELISA were performed at the WHO Reference Laboratory for Chagas' disease serology at the Federal University of Goiás, Brazil. The authors cross-referenced other publications (Camargo *et al*., 1973[75]; de Andrade *et al*., 1992[76]; Voller *et al*., 1975[72]) for the methods. The Applicant included the cross-referenced publications as well as provided brief details of the methods used for serological testing in the Laboratory standardization manual.[68] The differences noted in the information available from the cross-referenced publications compared to that in the Laboratory standardization manual[68] are summarized in Appendix-3, Table B. These differences can alter the performance of the test. There were no data available to support the intra-assay and inter-assay variability, controls used, and the cut-offs used to characterize the subjects as seropositive or seronegative.

The non-conventional AT-CL-ELISA was performed at the Federal University of São Paulo, Brazil. This appears to be the same laboratory as that of the cross-referenced publication

[75] Camargo ME, Hoshino S and Siqueira GRV. Hemagglutination with preserved, sensitized cells; a practical test for routine serologic diagnosis of American trypanosomiasis. *Revista do Instituto de Medicina Tropical de São Paulo* (1973) 15: 81-85.

[76] de Andrade ALSS, Zicker F, Luquetti AO, Oliveira RM, Silva SA, Souza JMP, Martelli, CMT. Surveillance of *Trypanosoma cruzi* transmission by serological screening of school children. Bull World Health Organ (1992) 70: 625-629.

(Almeida *et al*., 1994[77]; 1997[78]). The AT antigen used in the assay is a purified mucin-like trypomastigote glycoproteins. The glycoproteins are targeted by lytic anti-α-Gal antibodies, whose presence is thought to be associated with an active *T. cruzi* infection. This is based on studies by Krettli *et al* (1982[79]), that reported lytic antibodies by CoML assay; the lytic antibodies against the live trypomastigotes were present in all untreated subjects as well as subjects treated with either BZN or nifurtimox that were seropositive by conventional serological tests; however, these lytic antibodies were not detected in subjects that were negative by xenodiagnosis. Based on unpublished information, the authors stated that antibodies by CoML and IFA were detected in mice with active infection; however, in mice that were inoculated with dead *T. cruzi* antigen, antibodies were detected by IFA but not by CoML assay suggesting lytic antibodies measured by CoML to be an indicator of active infection. No cross-reactivity was observed by AT-CL-ELISA at a dilution of 1:2000, with fungal infection or healthy individuals vaccinated with *Neisseria meningitidis* polysaccharides A and C. Any possibility of cross-reactivity with leishmaniasis was not specified.

**Results:**

There is a lot of variability in antibody titers by all the four serological tests. The results show a trend towards a decrease in antibody titers as well as the number of subjects that became seronegative in BZN group compared to the placebo group by the four tests (Table 17 and Figure 28). The difference in the percentage of subjects that became seronegative, in the untreated group compared to BZN group, was significant by IHA and AT-EL-ELISA and not by the other serological tests (IFA and conventional ELISA).

Table 17: Study 2 (de Andrade *et al*., 1996[2]) - Seronegative subjects by different serological tests at baseline and Month 36

| **Test** | **Treatment arm** | **Visit Number (%)** | |
|---|---|---|---|
| | | **Baseline** | **Month 36** |
| **IHA*** | Placebo (n=65) | 0 (0.0) | 0 (0.0) |
| | Benznidazole (n=64) | 0 (0.0) | 9 (14.1) |
| **IFA** | Placebo (n=65) | 0 (0.0) | 0 (0.0) |
| | Benznidazole (n=64) | 0 (0.0) | 3 (4.7) |
| **Conventional ELISA** | Placebo (n=65) | 0 (0.0) | 0 (0.0) |
| | Benznidazole (n=64) | 0 (0.0) | 4 (6.3) |
| **AT-CL-ELISA*** | Placebo (n=65) | 0 (0.0) | 3 (4.7) |
| | Benznidazole (n=64) | 0 (0.0) | 35 (54.7) |
| **ECG** | Placebo (n=65) | 7 (10.8) | ND |
| | Benznidazole (n=64) | 6 (9.4) | ND |

Patients with a missing value were imputed as seropositive. EOT=end of treatment.
*Statistically significant compared to placebo (for details see statistics review by Dr Felicia Griffin).

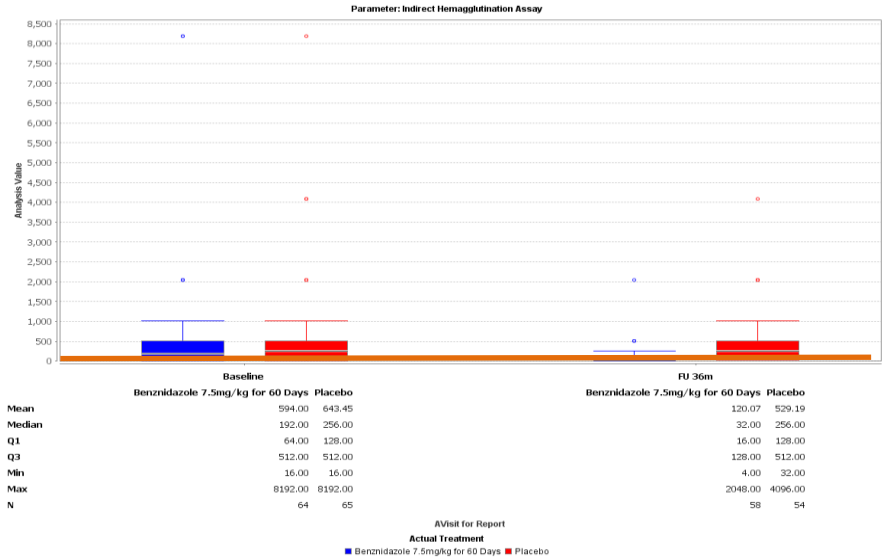
[77] Almeida IC, Ferguson MA, Schenkman S, and Travassos LR. Lytic anti-alpha-galactosyl antibodies from patients with chronic Chagas' disease recognize novel O-linked oligosaccharides on mucin-like glycosyl-phosphatidylinositol-anchored glycoproteins of *Trypanosoma cruzi. Biochem J* (1994) 304 (Pt 3): 793-802.

[78] Almeida IC, Covas DT, Soussumi LMT, and Travassos LR. A highly sensitive and specific chemiluminescent enzyme-linked immunosorbent assay for diagnosis of active *Trypanosoma cruzi* infection. *Transfusion* (1997) 37: 850-857.

[79] Krettli AU, Cancado JR, and Brener Z. Effect of specific chemotherapy on the levels of lytic antibodies in Chagas's disease. *Trans Roy Soc Trop Med Hyg* (1982) 76 (3): 334-340.

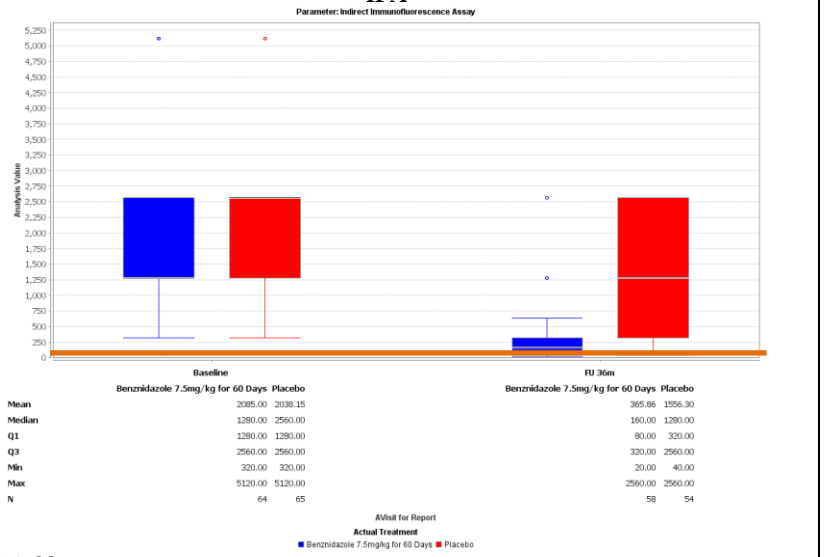
Figure 28: Study 2 (de Andrade *et al*., 1996[2]) – Antibody titers (Box whisker plots: Median and % quartile represented by bars) by IHA, IFA, conventional ELISA and AT-CL-ELISA at baseline and follow-up (Month 36).

**IHA**



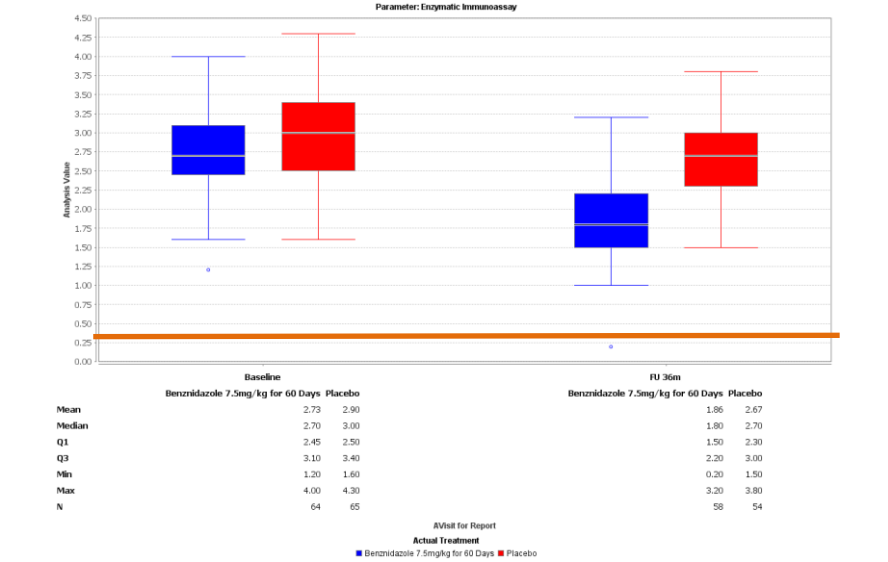

≥1/16 dilution represent positive

**IFA**



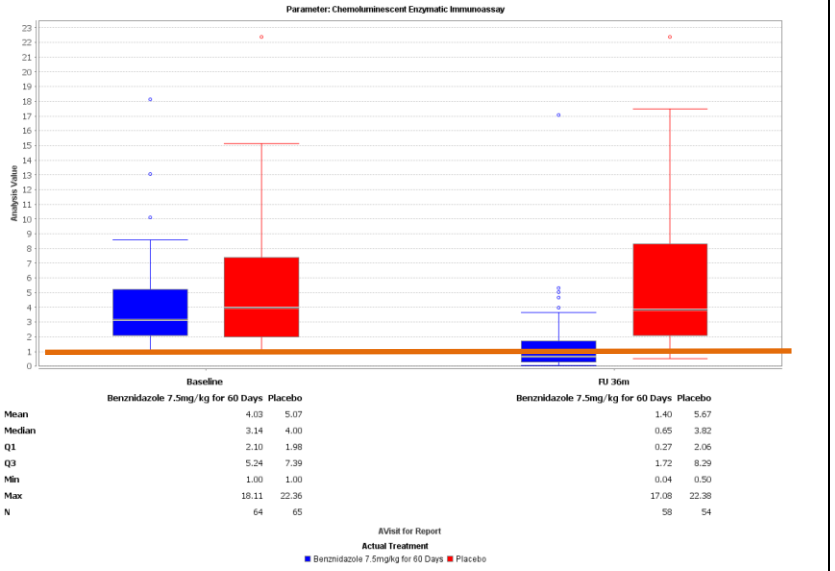

≥1/40 dilution represent positive

**Conventional EIA**




An OD≤0.4 was considered negative. Children had to show an ELISA index of 1.2 to be considered seropositive

**AT-EL-ELISA**




The cut-off was defined as "ten times negative control mean minus background control mean for each plate" and "positive serum samples gave ratios greater than 1.0"

*Comments:*
*Children positive by three serological tests (IHA, IFA, and ELISA) were enrolled. This is in accordance with the CDC and WHO recommendations to use at least 2 or 3 serological tests, respectively, to strengthen the validity of the obtained results for diagnosis of patients with Chagas disease. The vector control measures were implemented throughout the trial; therefore, possibility of re-infection was minimized.*

*The antibody response was evaluated by 4 different serologic tests (IHA, IFA, conventional ELISA, and AT-CL-ELISA). Conventional serological tests were performed at the WHO Reference Laboratory for Chagas disease serology at the Federal University of Goiás, Brazil; the non-conventional AT-CL-ELISA was performed at the Federal University of São Paulo R Sena Madureira, São Paulo, Brazil. This is the same laboratory as that of the cross-referenced publication (Almeida et al., 1997[78]). The Applicant provided some details of the different methods as well as the cut-offs that were used for characterizing the test results as seropositive or seronegative. However, the data supporting the performance characteristics of the assay were not available for review. The authors cross-referenced other publications for the methods. For the conventional serological tests, some differences were noted in the information available from the cross-referenced publications compared to that in the Laboratory standardization manual; such differences can alter the performance of the test. For the AT-CL-ELISA, testing was done in the same laboratory where the test was initially developed. However, no data were available to support the cut-offs used to characterize a subject as seropositive or seronegative.*

*The AT antigen used in the non-conventional ELISA is a glycosylinositol phospholipid-anchored glycoconjugates from trypomastigotes (F2/3), that is further purified and is thought to measure active T. cruzi infection. Most of the lytic antibodies in Chagas patients have a specificity for a-galactosyl-containing epitopes that are present in the AT (F2/3) antigen of trypomastigotes, but not in similar glycoproteins from epimastigotes and metacyclic forms. These antibodies, rather than most of the other antibodies, disappear sooner after elimination of the parasite from the infected host.*

*There is a lot of variability in antibody titers by the four serological tests used. The results show a trend towards a decrease in antibody titers as well as the number of subjects that became seronegative in BZN group compared to the placebo group by the four tests. Such differences are statistically significant by the AT-CL-ELISA and IHA but not by the other two tests (IFA and conventional ELISA). It is unclear if this is due to the difference in the antigens used for the different assays. The clinical relevance of the test results was not reported in this study.*

*Another study by the same group (Glavao et al., 2003[80]) reported the effect of BZN treatment on parasitemia based on detection of ≈330-bp T. cruzi kDNA fragment in paired samples (baseline and 3-year follow-up), measured by PCR, from 111 seropositive children, who were enrolled in a randomized trial. The trial design was the same as summarized above for Study 2 (de Andrade et al., 1996[2]) except that subjects were followed until 1997 and not until 1995. For PCR, preparation of DNA, amplification of the kDNA fragment, and slot blot hybridization was performed in EDTA containing blood in the Laboratorio de Biologia do Trypanosoma cruzi, Departamento de Parasitologia, Instituto de Ciencias Biologicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. The details of the method and performance*

[80] Galvao LMC, Chiari E, Macedo AM, Luquetti AO, Silva SA, and Andrade ALSS. PCR assay for monitoring *Trypanosoma cruzi* parasitemia in childhood after specific chemotherapy. *J Clin Micro* (2003) 41 (11): 5066-5070.

*characteristics of the assay used were not available for review. Receiver operating characteristic (ROC) curves were constructed by plotting sensitivity against 1-specificity and used to compare the performances of the three conventional serology tests (IFA, IHA, and ELISA), after a 3-year-follow-up, in BZN treated and untreated individuals. The best predictor was the test giving the highest area under the curve (Az); the Az was similar for all the three serological tests (Figure 29).*

Figure 29: ROC curve of IFA titers, IHA titers, and ELISA index for the identification


*At baseline, the relative sensitivity of the PCR was about 84%. The percentage of positive PCR results in the BZN treated group 3 years after treatment was significantly lower (P=0.01) than that in the placebo group (Table 18). Overall, the study suggests lower PCR positivity in the BZN treated subjects compared to placebo. These results should be interpreted with caution as a single negative does not mean the patient is cured. There is a possibility that waves of parasitemia may be intermittent and may recur during the long course of Chagas disease. The clinical relevance of this finding is not known.*

Table 18: PCR results for *T. cruzi*-infected children 3 years after BZN or placebo treatment


*Another published study by the same group (de Andrade et al., 2004[81]) reported a six-year follow-up of the subjects in the BZN and placebo group. Of the 58BZN treated children, 37 were seronegative and 21 seropositive at Month 36. Of the 37 seronegative subjects, 33 (89.2%)*

[81] de Andrade ALSS, Martelli, CMT, Oliveira RM, Silva SA, Aires AIS, Soussumi LMT, Covas DT, Silva LS, Andrade JG, Travassos LR, and Almeida IC. Short report: benznidazole efficacy among T*rypanosoma cruzi* −infected adolescents after a six-year follow-up. *Am J Trop Med Hyg* (2004) 71 (5): 594-597.

*remained seronegative by AT-CL-ELISA at 6 year follow-up; 14 (66.7%) of the seropositive subjects became seronegative. The authors state that "In the placebo group, of 52 individuals who were seropositive after three years of follow-up, 32 remained positive after six years, whereas 11 became seronegative"; the serological findings for the remaining 9 subjects were not specified. Overall, the results show that at 6 year follow-up, seronegative conversion occurred in nearly 90% of the BZN treated patients while the antibody titers were stable among untreated individuals.*

*The authors state that no incident case of ECG abnormality was found in this extended evaluation, although early development of cardiomyopathy (complete right bundle branch block) was detected in five children (four in the placebo group) after three years of treatment. It is unclear whether the subjects that showed cardiac abnormalities were seropositive or seronegative.*

**4.3. Study 3 (Molina *et al*., 2014)**

Molina *et al*., 2014[3] reported the results of a prospective randomized open-label trial in adults (≥18 years of age) with chronic *T. cruzi* infection; the study was conducted between 2010 and 2012 at three centers participating in the International Health Program of the Catalan Institute of Health (PROSICS; Protocol Number CHAGASAZOL01). The inclusion criteria included verification of *T. cruzi* infection by two different serologic tests and positive result of RT-PCR. Exclusion criteria included previous treatment for Chagas disease, plans for travel during the follow-up period where the disease was known to be endemic, and subjects with immunosuppression. The subjects were randomized to 3 treatment arms and treated orally with either low-dose (100 mg, bid; n=26) posaconazole, high-dose (400 mg, bid; n=27) posaconazole or BZN (Lafepe product: 150 mg, bid; n=26) for 60 days. Patients who had chronic *T. cruzi* infection but no clinical, radiologic, or electrocardiographic evidence of visceral involvement were characterized as having an indeterminate form of Chagas disease. There were 51 (65%) patients with indeterminate disease, 17 (22%) cardiac involvement, 5 (6%) gastro-intestinal involvement, and 5 (6%) involvement of more than one organ system (mixed form). In the BZN group, treatment was discontinued in 5 patients because of severe cutaneous reactions.

**Primary endpoint**

Consistently negative results by RT-PCR assay, for detection of *T.* cruzi DNA, over the entire follow-up period.

**Secondary end points**

- The incidence of serologic conversion from positive to negative or any variation in the absorption index (the amount of light that is absorbed by the serum, as compared with a standard control) on serologic ELISA, indicating some treatment activity at the end of the follow-up period.
- The percentage of patients who discontinued treatment because of adverse events.

The anti-parasitic activity was based on the presence of *T. cruzi* DNA in blood, using RT-PCR assay during the treatment period (Days 7, 14, 28, 45, and 60) and at Months 2, 4, 6, and 10 after the end of treatment. The RT-PCR assay was performed based on the amplification of a 166 bp

segment in the satellite DNA of *T. cruzi* by the method of Piron *et al*., 2007[82]. The testing was performed in [redacted](b) (4) [redacted](b) (4). All the details of the method and performance of the assay were not available for review. Based on the information available in the publications, the sensitivity was 0.8 parasites/mL (50% positive hit rate) and 2 parasites/mL (95% positive hit rate); this is based on serial dilution of samples spiked with DNA from epimastigotes of the Maracay strain of *T. cruzi*. A negative result in the RT-PCR assay was reported inferior to 2.8 parasites/mL. For quantitative results, the RT-PCR was performed in triplicate with 10-fold serial dilutions of DNA from blood spiked with $10^6$ epimastigotes/mL; a linear curve from $10^5$ down to 10 parasites/mL of blood, with a four-log dynamic range was observed. The results of the quantitative RT-PCR assay were not shown. None of the 124 *T. cruzi* seronegative samples or 20 samples from visceral leishmaniasis patients tested positive by RT-PCR. The authors compared the performance of the RT-PCR assay with a nested PCR by testing blood samples from 39 subjects with Chagas disease who were native to endemic areas (33 Bolivians, 3 Argentines, 1 Brazilian, 1 Honduran) and living in Barcelona. The results were comparable.

Serological testing was performed at baseline as well as at Months 6 and 10 after the end of treatment by two commercial ELISAs [the Bioelisa Chagas – non-conventional using recombinant antigens consisting of 4 epitopes (Biokit, Spain) and the *T. cruzi* ELISA (Ortho-Clinical Diagnostics, USA)- conventional]. All tests were performed at the Infectious Disease Department of the Hospital University Vall d´Hebron. The information available from the package inserts for the two commercially available tests is summarized in Appendix-3, Table C.

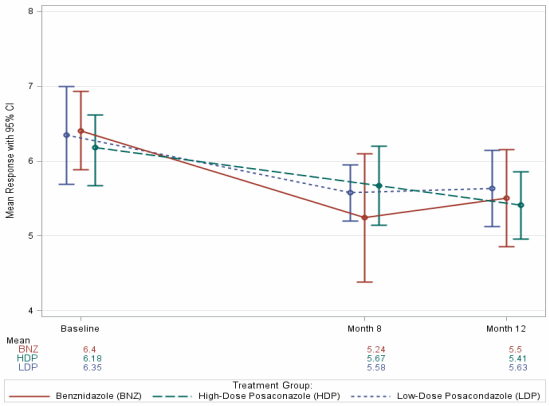
**Results:**
On Day 7 after initiation of treatment, 19 of the 26 subjects (73.1%) in the BZN treated group were PCR negative; on Day 14 of treatment, a majority of the subjects were PCR negative and remained negative until the end of follow-up i.e., Month 12 (Figure 30 and Table 19). However, all subjects remained seropositive (Table 19) and there was no significant change in antibody titers by either of the ELISA methods at follow-up visits compared to the pretreatment levels (Figure 30); this could be due to a short duration of follow-up and the serological tests used.

[82] Piron M, Fisa R, Casamitjana N, Lopez-Chejadeet P, Puig L, Verges M, Gascon J, Gomez I Prat J, Portus M, and Sauleda S. Development of a real-time PCR assay for *Trypanosoma cruzi* detection in blood samples. *Acta Trop* (2007) 103: 195-200.

Figure 30: Study 3 (Molina *et al*., 2014[3]) – Antibody titers by conventional and non-conventional ELISAs at baseline and follow-up visits as well as .

**ELISA: conventional**

Ortho-Clinical Diagnostics, USA – *T. cruzi* lysate

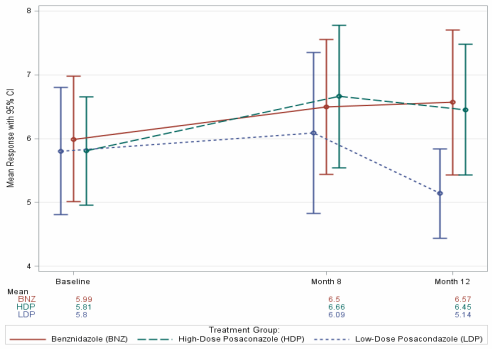



The cut-off is defined here as mean OD of the positive calibrator multiplied by 0.425. Results represent mean±SD.

**ELISA:non-conventional**

Bioelisa Chagas (Biokit, Spain)

Recombinant antigen consisting of four immunodominant epitopes of *T. cruzi*




The cut-off value is defined as mean absorbance of the negative controls plus 0.300 and the sample is positive if the absorbance divided by the cut-off gives a ratio over 1.0. Results represent mean±SD.

**RT-PCR - Kaplan-Meier plot of time to reversion to PCR positive**

The horizontal axis shows number of days from the Day 60 evaluation. The vertical axis shows the proportion of patients who remain PCR negative. Patients with all missing measurements or at least one missing measurement and all other measurements recorded as negative at any time point after Day 60 are censored in this plot. Each step indicates a reappearance of parasite in blood samples, i.e., a PCR positive response. The plot includes only those ITT patients who were PCR negative at Day 60.

Figures constructed by Dr Janelle Charles, PhD, Statistics Reviewer

*Comments:*

*Overall, the study suggests that treatment with BZN converted higher percentage of subjects from PCR positive to PCR negative; quantitation of DNA was not performed. A decrease in the number of PCR positive subjects was observed by Day 7 of treatment with BZN. The results of PCR should be interpreted with caution; a positive PCR finding may be a marker for treatment failure. However, a negative PCR result may be indicative only of the absence of circulating DNA at the moment when blood is drawn for testing. The clinical relevance of these findings is unclear.*

*Serological testing was performed by two ELISA tests. One of the ELISA test used was a conventional ELISA that is FDA cleared; the other ELISA is commercially available in Spain and is a non-conventional ELISA using 4 recombinant epitopes of T. cruzi. There was no change in number of subjects that were seropositive by any of the serological tests. This may be due to the short term follow-up in this study.*

Table 19: Study 3 (Molina *et al*. 2014[3])-Number (%) seronegative or RT-PCR negative subjects by different serological tests at baseline, end of treatment (EOT), and follow-up.

| Test | Treatment arm | Visit [number of subjects negative (%)] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Baseline | During treatment | | | | | Follow-up | | |
| | | | Day 7 | Day 14 | Day 28 | Day 45 | Day 60 | Month 4 | Month 6 | Month 8 | Month 12 |
| ELISA - conventional<br><br>Ortho-Clinical Diagnostics, USA – *T. cruzi* lysate | Posa low dose (n=26) | 0 (0.0) | ND | ND | ND | ND | ND | ND | ND | 0 (0.0) | 0 (0.0) |
| | Posa high dose (n=27) | 0 (0.0) | ND | ND | ND | ND | ND | ND | ND | 0 (0.0) | 0 (0.0) |
| | Benznidazole (n=45) | 0 (0.0) | ND | ND | ND | ND | ND | ND | ND | 0 (0.0) | 0 (0.0) |
| ELISA – non-conventional Recombinant antigen consisting of four immunodominant epitopes of *T. cruzi*<br><br>Bioelisa Chagas (Biokit, Spain) - | Posa low dose (n=26) | 0 (0.0) | ND | ND | ND | ND | ND | ND | ND | 0 (0.0) | 0 (0.0) |
| | Posa high dose (n=27) | 0 (0.0) | ND | ND | ND | ND | ND | ND | ND | 0 (0.0) | 0 (0.0) |
| | Benznidazole (n=26) | 0 (0.0) | ND | ND | ND | ND | ND | ND | ND | 0 (0.0) | 0 (0.0) |
| RT-PCR | Posa low dose (n=26) | 0 (0.0) | 13 (50.0) | 17 (65.4) | 20 (76.9) | 18 (69.2) | 18 (69.2) | 11 (42.3) | 6 (23.1) | 7 (26.9) | 6 (23.1) |
| | Posa high dose (n=27) | 1 (3.7) | 18 (66.7) | 22 (81.5) | 27 (100) | 26 (96.3) | 26 (96.3) | 19 (70.4) | 16 (59.3) | 12 (44.4) | 8 (29.6) |
| | Benznidazole (n=26) | 0 (0.0) | 19 (73.1) | 24 (92.3) | 24 (92.3) | 21 (80.8) | 22 (84.6) | 20 (76.9) | 19 (73.1) | 18 (69.2) | 16 (61.5) |

Patients with a missing value were imputed as seropositive or PCR positive
EOT=end of treatment; ND=not done

**4.4. Study 4 (DNDi-CH-E1224-001)**

This was a randomized, double-blind, placebo-controlled phase 2 study to evaluate the efficacy and safety of E1224 (a prodrug of ravuconazole) and BZN (Lafepe product) in 231 adult patients, between the age of 18 and 50 years, with chronic indeterminate Chagas disease. The study took place in two research centers in Tarija and Cochabamba, Bolivia between 2011 and 2012. The participants had to be diagnosed of *T. cruzi* infection by two out of three positive serological tests comprising of conventional ELISAs, IFA, and IHA. If two serological tests were positive (qualitative), quantitative RT-PCR testing was performed over 7 days (two blood samples on first day of the screening and one sample after 7 days); at least one positive PCR result was necessary for enrolment. The subjects should not have had any signs and/or symptoms of the chronic cardiac and/or digestive form of Chagas disease. The patients were randomized to 5 different treatment arms:

- E1224 - high dose (400 mg/week; 8 weeks).
- E1224 - low dose (200 mg/week; 8 weeks).
- E1224 - short dose (400 mg/week; 4 weeks plus 4 weeks placebo).
- Benznidazole - (5 mg/kg/day; 8 weeks).
- E1224 matching placebo group.

Primary efficacy endpoint was based on PCR findings at the end of treatment. Subjects were treated for 8 weeks and follow-up period was 10 months. The efficacy was measured by repeated qualitative and quantitative PCR as well as serological tests.

***Serological and parasitological testing***

As stated above, for screening at the beginning of the study, serological testing was performed by the conventional ELISAs (b) (4) IFA, and IHA (b) (4) The baseline samples as well as those collected during the treatment and follow-up periods, testing was performed by the Chagatek conventional ELISA from (b) (4)), recombinant ELISA (b) (4) (b) (4) and AT-CL-ELISA (Almeida *et al*., 1997[78]; de Andrade *et al*., 1996[2]; Izquierdo *et al*., 2013[83]). The two of the serological tests used were commercially available. However, none of the tests are FDA cleared. The assay information available from the test package insert, laboratory manual or the cross-referenced publications as well as the cut-offs used to characterize a patient as positive or negative is summarized in Appendix-3, Table D.

The RT-PCR assay was performed at baseline as well as during treatment and follow-up period. The assay was based on the amplification of a 166 bp segment in the satellite DNA of *T. cruzi* by the method of Piron *et al*., 2007[82]; Schijman *et al* ., 2011[84]; Duffy et *al*., 2013[85]; Bisio *et al*., [86];

[83] Izquierdo L, Marqwues AF, Gállego M, Sanz S, Tebar S, Riera C, Quintó L, Aldasoro E, Almeida IC, and Gascon J. Evaluation of a chemiluminescent enzyme-linked immunosorbent assay for the diagnosis of *Trypanosoma cruzi* infection in a nonendemic setting. *Mem Inst Oswaldo Cruz, Rio de Janeiro* (2013) 108 (7): 928-931.

[84] Schijman AG, Bisio M, Orellana L, Sued M, Duffy T, Jaramillo AMM, Cura C, Auter F, Veron V, Qvarnstrom Y, Deborggraeve S. Hijar G, Zulantay I, Lucero RH, Velazquez E, Tellez T, Leon ZS, Galvao L, Nolder D, Rumi MM, Levi JE, Ramirej JD, Zorilla P, Flores M, Jercic MI, Crisante G, Anez N, de Castro AM, Gonzalez CI, Viana KA, Yachelini P, Torrico F, Robello C, Diosque P, Chanvez OT, Aznar C, Russomando G, Buscher P, Assal A, Guhl F, Sosa-Estani S, DaSilva A, Britto C, Luquetti A, and Ladzins J. International study to evaluate PCR methods for detection of *Trypanosoma cruzi* DNA in blood samples from Chagas disease patients. *PLoS Negl Trop Dis* (2011) 5: e931.

Ramirej *et al*., 2015[87]). The Applicant provided the details of the method used. The RT-PCR was developed using DNA from parasites as positive controls spiked into blood – the gold standard of RT-PCR development. Additionally, external quality assurance program was implemented. The Applicant states that there was high congruence between the laboratories (for details see Appendix-3, Table E). Blood samples were collected in triplicate at most of the visits.

**Results:**

The results suggest a reduction in parasitic load, measured by RT-PCR, in subjects treated with BZN compared to placebo at the end of treatment (Day 65) and follow-up visits until Month 12 (Figure 31). The number of patients that were positive by RT-PCR at different visits was lower in the BZN treated group compared to the placebo group (Table 20). Of the 40 subjects in the BZN group that were negative by RT-PCR on Day 8, 6 were positive at one or more visits for the duration of the study (Day 36, 65, Month 6 or 12). Of the 15 subjects in the placebo group that were RT-PCR negative at Day 8, 13 were positive at one or more visits for the duration of the study; two subjects remained RT-PCR negative for the duration of study. It is unclear if RT-PCR positivity after treatment is due to intermittent release of DNA or limitation of the PCR assay.

There was no difference in the number of subjects that were seropositive by any of the assays in both groups; all subjects remained antibody positive (Table 20). However, there is a decline in antibody titers in both groups (Figure 31). By AT-EL-ELISA, the antibody titers were lower in the BZN treated subjects compared to placebo.

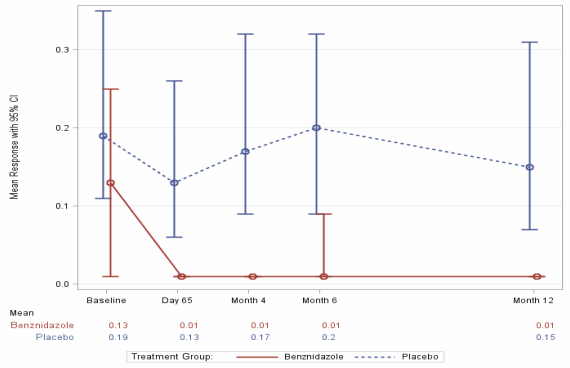
[85] Duffy T, Cura CI, Ramirej JC, Abate T, Cayo NM, Parrado R, Bello ZD, Velazquez E, Munoz-Calderon A, Juiz NA, Basile J, Garcia L, Riarte A, Nasser JR, Ocampo SB, Yadon ZE, Torrico F, de Noya BA, Ribeiro I, and Schijman AG. Analytical Performance of a Multiplex Real-Time PCR Assay Using TaqMan Probes for Quantification of *Trypanosoma cruzi* Satellite DNA in Blood Samples. *PLoS Neglected Tropical Diseases* (2013) 7: e2000.

[86] Bisio M, Orellana L, Duffy T, Garcia R, Sued M, Abate T, Schijman AG and Participants at the International workshop on "Standardization and validation of clinical use of PCR for *Trypanosoma cruzi* DNA detection in Chagas disease". Evaluation of DNA extraction methods for PCR-based detection of *Trypanosoma cruzi* DNA in blood specimens using an Internal Amplification Control. *American Journal of Tropical Medicine & Hygiene*. Manuscript submitted ID: AJTMH-12-0313.

[87] Ramírez JC, Cura CI, da Cruz Moreira O, Lages-Silva E, Juiz N, Velázquez E, Ramírez JD, Alberti A, Pavia P, Flores-Chávez MD, Muñoz-Calderón A, Pérez-Morales D, Santalla J, Marcos da Matta Guedes P, Peneau J, Marcet P, Padilla C, Cruz-Robles D, Valencia E, Crisante GE, Greif G, Zulantay I, Costales JA, Alvarez-Martínez M, Martínez NE, Villarroel R, Villarroel S, Sánchez Z, Bisio M, Parrado R, Maria da Cunha Galvão L, Jácome da Câmara AC, Espinoza B, Alarcón de Noya B, Puerta C, Riarte A, Diosque P, Sosa-Estani S, Guhl F, Ribeiro I, Aznar C, Britto C, Yadón ZE, Schijman AG. *J Mol Diagn* (2015) 17 (5): 605-615.

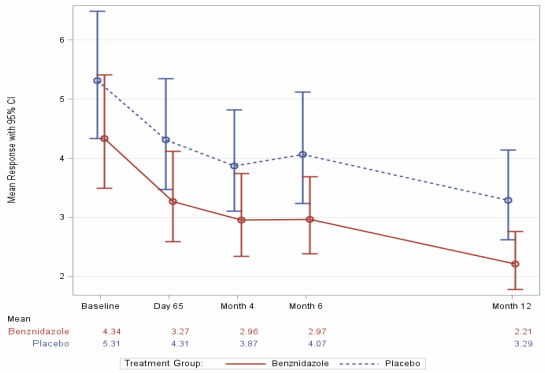
Figure 31: Study 4 (DNDi-CH-E1224-001) – Antibody titers by conventional and non-conventional ELISAs and parasitological observations by RT-PCR at baseline and follow-up visits.

**RT-PCR**




Results represent geometric mean.
The patient was RT-PCR positive when the CT value was <40. The limit of quantification was 1.5 parasite equivalents/mL.

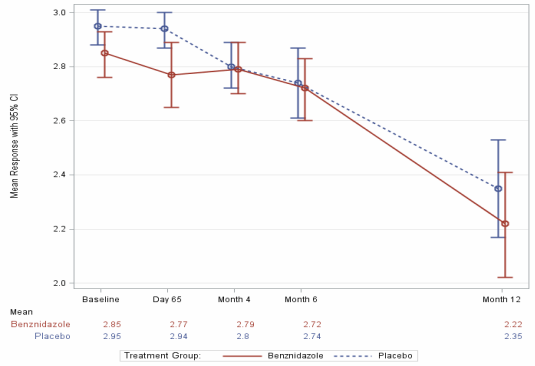
**AT-CL-ELISA – non-conventional**




Results represent geometric mean.
Cut-off values defined as the upper prediction limit, which is expressed as the standard deviation multiplied by a factor based on the number of negative controls and the confidence level (99.5%)

**ELISA - conventional**

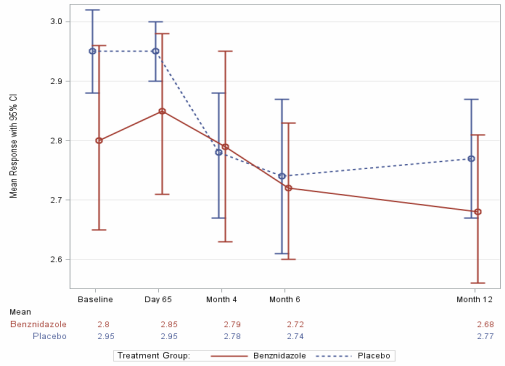
(b) (4)




Results represent mean.
The cut-off defined as the average of the negative control OD readings plus 0.200.

**Recombinante ELISA – non-conventional**

(b) (4)




Results represent those recorded on CRF; testing was done of all the stored samples as well. The mean values were slightly lower in stored samples compared to those tested in real time; however, the trend was same. The cut-off defined as average negative control readings plus 0.300 OD.

Figures constructed by Dr Janelle Charles, PhD, Statistics Reviewer

Table 20: Study 4 (DNDi-CH-E1224-001)- Number (%) seronegative or RT-PCR negative subjects by different serological tests at baseline, end of treatment (EOT), and follow-up at Months 3, 6, 12, 24 and 48.

| Test | Treatment arm | Visit [number of subjects negative (%)] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Baseline | During treatment | | | Follow-up | | | |
| | | | Day 8 | Day 15 | Day 36 | Day 65 | Month 4 | Month 6 | Month 12 |
| **ELISA - conventional** | Placebo (n=47) | 0 (0.0) | ND | ND | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| **Chagatek** (b) (4) | Benznidazole (n=45) | 0 (0.0) | ND | ND | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| **Recombinant ELISA** | Placebo (n=47) | 0 (0.0) | ND | ND | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| **non-conventional** (b) (4) | Benznidazole (n=45) | 0 (0.0) | ND | ND | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| **RT-PCR** | Placebo (n=47) | 0 (0.0) | 15 (31.9) | 16 (34.0) | 8 (17.0) | 12 (25.5) | 9 (19.1) | 6 (12.8) | 10 (21.3) |
| | Benznidazole (n=45) | 0 (0.0) | 40 (88.9) | 43 (95.6) | 43 (95.6) | 41 (91.1) | 43 (95.6) | 41 (91.1) | 42 (93.3) |

Patients with a missing value were imputed as seropositive or PCR positive
Of the 40 subjects in the BZN group that were negative by RT-PCR on Day 8, 6 were positive at Day36, 65, Month 6 or 12. Of the 15 subjects in the placebo group that were RT-PCR negative at Day 8, 13 were positive at one or more visits for the duration of the study; two subjects remained RT-PCR negative for the duration of study.
EOT=end of treatment; ND=not done

*Comments:*
*Overall, the study suggests that treatment with BZN decreased parasitic load, measured by RT-PCR, and converted higher percentage of subjects from PCR positive to PCR negative. The results of PCR should be interpreted with caution. Although a positive PCR finding may be a marker for treatment failure, however, a negative PCR result may be indicative only of the absence of circulating DNA at the moment when blood is drawn for testing. The clinical relevance of these findings is unclear.*

*Serological testing was performed by three ELISA tests. None of the tests used are FDA cleared; two of the ELISAs are commercially available in Argentina. All subjects remained seropositive by all the serological tests. This may be due to the short term follow-up in this study. By AT-EL-ELISA, there is a trend towards a decrease in antibody titers in subjects treated with BZN compared to the untreated group.*

### 4.5. Study 5 (Viotti *et al.*, 2006)

Viotti *et al.*, 2006[4] reported the long-term outcomes in 566 adults (30 to 50 years of age) with Chagas disease. This was a placebo controlled open-label non-randomized trial in subjects with positive results by 3 serologic tests and no clinical signs of heart failure. Subjects were evaluated for clinical and laboratory parameters at the Chagas Disease Section at Hospital Eva Peron, Buenos Aires, Argentina. Patients older than 50 years of age were excluded to avoid misinterpretation of electrocardiographic changes; patients younger than 30 years of age were excluded because patients in this age group rarely present to the center. The clinical evaluations included medical history, electrocardiography, chest radiography, and echocardiography. Serological testing was done by CFT, IHA, IFA, or conventional ELISA, performed at the reference center, Instituto Nacional de Parasitologia Dr. Mario Fatala Chaben. (b) (4) (b) (4)

The Applicant states that the IHA, IFA, and ELISA methods used were the same as those used for Study 1.

Patients were stratified according to the Kuschnir's clinical classification (Table 21). BZN, 5 mg/kg/day, was administered orally for 30 days. Follow-up of clinical and serological response was conducted up to ~15 years (median 9.8 years). The primary outcome was disease progression, defined as a change to a more advanced Kuschnir group or death. Secondary outcomes included new abnormalities on electrocardiography and serologic reactivity.

Table 21: Study 5 (Viotti *et al.*, 2006[4]) - Patient characteristics and progression of disease at the end of study

| Group | Baseline characteristics | | | | Patients that progressed* | |
|---|---|---|---|---|---|---|
| | Serology | Electrocardiography | Chest radiography | Cardiac enlargement / signs of heart failure | Placebo n/N (%) | Benznidazole n/N (%) |
| 0[1] | + | Normal | Normal | None | 13/180 (7.2) | 6/180 (3.3) |
| I | + | Abnormal | Normal | None | 14/75 (18.7) | 3/73 (4.1) |
| II | + | Abnormal | Abnormal | Yes/None | 13/28 (46.4) | 3/30 (10.0) |
| III | + | Abnormal | Abnormal | Yes/Yes | Not specified | Not specified |
| **Subjects changed clinical group to a more severe Kuschnir group at follow-up** | | | | | 40/283 (14.1) | 12/283 (4.2) |
| **Seronegative by 3 tests at follow-up**** | | | | | 12/212 (5.7)[#] | 32/218 (14.7)[^] |
| **Mortality** | | | | | 12/283 (4.2) | 3/283 (1.1) |

[1]Represent indeterminate stage disease
*Treated and untreated patients that changed clinical group to a more severe Kuschnir group at follow-up
**Complete seronegative status was achieved in a median of 11.7 years
[#]Of 12 seropositive patients, 9 were in Group 0 and 3 in Group 1.
[^]Of the 32 seropositive patients, 19 were in Group 0, 11 in Group 1 and 2 in Group 2.

The results showed that the proportion of patients that changed clinical group to a more severe Kuschnir group were lower in the BZN treated group compared to placebo. Also, higher number of subjects became seronegative in the BZN group compared to placebo (Table 21). The authors state that none of subjects who achieved complete negative results on serologic testing changed clinical group during the follow-up, regardless of treatment.

The mortality rate was lower (1.1%) in the BZN treated group compared to the untreated group (4.2%); causes of death were stated to be sudden and include heart failure (for more details see clinical review).

*Comments:*
*Overall, the study suggests that BZN treatment of patients with indeterminate stage Chagas disease may decrease the risk for disease progression. BZN treatment was associated with a reduced risk for progression of Chagas heart disease and an increased rate of seroconversion.*

## 5. INTERPRETIVE CRITERIA/BREAKPOINTS

The Applicant has not requested any interpretive criteria in the labeling. This is appropriate as the tests to measure *in vitro* susceptibility of *T. cruzi* parasites are not standardized and their use is limited to research laboratories.

## 6. THE LABELING

### 6.1. Applicant's version of the microbiology section of the labeling

**12.1 Mechanism of action**
TRADENAME is (b) (4) drug. *[see* (b) (4) *(12.4)]*

**12.4 Microbiology**
Mechanism of Action
Benznidazole (b) (4).
(b) (4)

Reference (b) (4)

Resistance

(b) (4)

Antimicrobial Activity

(b) (4) TRADENAME against (b) (4) (b) (4) *T. cruzi* strains.

***6.2. Comments***

- *Changes are recommended in Section 12.4 subheading 'Mechanism of action' for clarity and accuracy of the available information. The studies show that BZN inhibits the synthesis of DNA, RNA, and proteins within the parasite. BZN is reduced by Type I NTR present within the T. cruzi parasites to a series of metabolites that can damage DNA and other macromolecules. However, the precise mechanism of action is not known. It will be useful to add information on activity of BZN against mammalian cells.*

- *Under the subheading 'Antimicrobial activity' the Applicant has proposed to state that* (b) (4) (b) (4) (b) (4) *For more accurate representation of data, it is recommended that the following should be stated:*

  *Benznidazole is active against Trypanosoma cruzi. However, the sensitivity of T. cruzi strains, from different geographic regions, to benznidazole may vary.*

- *Under the subheading 'Resistance' the Applicant proposed* (b) (4) (b) (4) (b) (4) (b) (4) *It should be stated that there is a potential for development of resistance to benznidazole. The information available on the mechanisms of resistance should be included.*

**6.3. FDA's version of the labeling**
Additions marked as double-underlined and deletions as striked out

**12.1 Mechanism of action**
(b) (4)-Benznidazole is (b) (4) drug. *[see* (b) (4) *Microbiology (12.4)]*

**12.4 Microbiology**
Mechanism of Action
Benznidazole-(b) (4)-, a nitroimidazole, is an antiprotozoal drug. Benznidazole inhibits the synthesis of DNA, RNA, and proteins within the *T. cruzi* parasite. Studies suggest that benznidazole is reduced by a Type I nitroreductase (NTR) enzyme of *T. cruzi* producing a series of short-lived intermediates that may promote damage to several macromolecules including DNA. In mammalian cells, however, benznidazole is metabolized by reduction of the nitro group to an amino group by a Type II NTR enzyme. The precise mechanism of action is not known.

(b) (4)

(b) (4)

[redacted]

Antimicrobial Activity

(b) (4)

[redacted]

(b) (4) Benznidazole is active against all three stages, trypomastigotes, amastigotes, and epimastigotes, of *Trypanosoma cruzi*. However, the sensitivity of *T. cruzi* strains to benznidazole, from different geographic regions, may vary.

Resistance

Studies *in vitro* and in mice infected with *T. cruzi* suggest a potential for development of resistance to benznidazole.

The mechanisms of drug resistance appear to be multifactorial. These mechanisms include decreased activity due to a mutation in the nitroreductase (*TcNTR*) gene. Other mechanisms include higher efflux activity due to over expression of $TcPGP_1$ and $TcPGP_2$ genes that encode p-glycoprotein as well as *TcABCG1* genes that encode ATP-binding casette transporters. Also, some studies reported overexpression of other genes *TcFeSOD-A* and *TcCyP19* that encode superoxide dismutase and cyclophilin, respectively, which have diverse biological function and may help parasite survival. However, the clinical relevance of these findings is not known.

*[See appended electronic signature page]*
Shukal Bala, Ph.D.
Microbiologist, DAIP

**CONCURRENCE:**
DAIP/Acting Deputy Director/ Dmitri Iarikov, MD, PhD
**CC:**
DAIP/PM/Gregory DiBernardo

# Appendix-1

## Summary of studies supporting the *in vitro* activity of BZN against *T. cruzi*

| Parasite stage (Reference) and experimental design | Strain (DTU) | $IC_{50}$ |
|---|---|---|
| *Epimastigote used for culture*<br>(Polak and Richle, 1978[28])<br>$5x10^6$ cells (mainly epimastigotes; about 20% trypomastigotes) were cultured in SGH medium with different concentrations of BZN and morphological evaluations performed at different time points for up to 3 days by phase contrast microscopy. Growth and viability of the parasite was reported. The BZN $IC_{95}$ was 60 µg/mL. | Y (II) | 5 µg/mL |
| *Epimastigote used for culture*<br>(Neal and van Bueren, 1988[32])<br>The epimastigotes were cultured in Schneider's insect culture medium and underwent >10 subcultures. The $10^6$ epimastigote parasites from the log phase were incubated with and without drug, at 26°C, for 4 to 12 days depending on the strain. The growth was measured by $^3$H-thymidine incorporation. The growth of different strains varied (mean generation time for Sonya clone-66 hours; Colombiana and Y strains-22 hours). The parasite stages present in culture varied (Colombiana-100% amastigotes; Sonya clone-50% mixture of epimastigotes and amastigotes; for other strains-100% epimastigotes). No flagellum or dividing amastigotes were observed by electronmicrography in Colombiana and Sonya clone cultures and uptake of $^3$H-thymidine was lowest. | Sonya clone* (not specified) | 1.80 µM<br>(0.47 µg/mL) |
| | Colombiana* (I) | 9.70 µM<br>(2.52 µg/mL) |
| | Tulahuen** (VI) | 1.60 µM<br>(0.42 µg/mL) |
| | Y** (II) | 2.20 µM<br>(0.57 µg/mL) |
| | Peru**<br>(not specified) | 7.40 µM<br>(1.93 µg/mL) |
| *Amastigote*<br>(Neal and van Bueren, 1988[32])<br>Mouse peritoneal macrophages ($0.175x10^6$) were infected with trypomastigotes ($0.18-0.25x10^6$); after 24 hours the medium was replaced and incubated with or without drug for 3 to 5 days. The numbers of infected and uninfected macrophages were counted.<br><br>The responsive strains (Tulahuen, Y and Peru) lysed macrophages in 3 days, whereas the nonresponsive strains (Sonya clone and Colombiana) required 4 to 5 days. Macrophage infections with the nonresponsive strains showed a lower infectivity (about 20-26%) compared to the responsive strains, which infected 40-90% of macrophages. | Sonya clone*<br>(not specified) | 1.99 µM<br>(0.52 µg/mL) |
| | Colombiana* (I) | 0.41 µM<br>(0.11 µg/mL) |
| | Tulahuen** (VI) | 0.09 µM<br>(0.02 µg/mL) |
| | Y** (II) | 2.58 µM<br>(0.67 µg/mL) |
| | Peru**<br>(Not specified) | 0.33 µM<br>(0.09 µg/mL) |
| *Epimastigote*<br>(Canavaci *et al.*, 2010[34])<br>Parasite lines expressing the firefly luciferase or the tandem tomato fluorescent protein (tdTomato) were cultured with and without drug for 4 days. Growth was measured microscopically as well as by flow cytometry (change in fluorescent intensity). | CL tdTomato | Microscopically<br>12.1 µM (3.15 µg/mL)<br>Flow cytometry<br>8.8 µM (2.29 µg/mL) |
| *Amastigote*<br>(Canavaci *et al.*, 2010[34])<br>Metacyclic trypomastigotes grown in gamma irradiated Vero cells and medium replaced. Different concentrations of BZN added and cultured for up to 4 days. The change in fluorescence intensity was determined. | CL tdTomato | ~0.16 µg/mL<br>(0.04 µg/mL) |
| *Epimastigotes*<br>(Moreno *et al.*, 2010[35])<br>Exponentially growing epimastigotes ($10^7$ parasites/mL) were incubated with the drug for 72 hours at 28 C and the number of living parasites counted. The stock solution of BZN was diluted in DMSO; the final DMSO concentrations of 0.031-4% had no significant effect on parasite growth of the epimastigotes of the VL10 strain.<br><br>#Represent strains susceptible - for which BZN susceptibility had been measured in infected mice. The strains were classified as follows:<br>• susceptible (cure rates > 66%) – Berenice 62 and CL.<br>• partially susceptible (cure rates between 33-66%) – Y.<br>• resistant (cure rates < 33%) - Colombiana, VL10 and YuYu (see shaded cells). | 115 (V) | 7.6 µM<br>(1.98 µg/mL) |
| | BE-62 (II)# | 12.8 µM<br>(3.33 µg/mL) |
| | CL Brener (VI)# | 13.6 µM<br>(3.54 µg/mL) |
| | Y (II)# | 16.3 µM<br>(4.24 µg/mL) |
| | BE-78 (II) | 15.8 µM<br>(4.11 µg/mL) |
| | B147<br>(Not determined) | 25.5 µM<br>(6.64 µg/mL) |
| | Colombiana (I)# | 25.4 µM<br>(6.61 µg/mL) |
| | Esmeraldo cl3 (II) | 26.7 µM<br>(6.95 µg/mL) |
| | Silvio X10 (I)# | 26.1 µM<br>(6.79 µg/mL) |
| | VL10 (II)# | 27.3 µM<br>(7.10 µg/mL) |
| | SC2005<br>(Not determined) | 32.1 µM<br>(8.35 µg/mL) |
| | YuYu (I)# | 32.0 µM<br>(8.33 µg/mL) |
| *Amastigote*<br>(Mejia *et al.*, 2012[36])<br>Transformed parasites were grown in Vero or rat skeletal myoblast L6 cells; details of the method not available. | 28 strains from different regions of Colombia (haplotypes I and II) | 1.5 – 34.6 µM<br>(0.39-9.00 µg/mL) |
| *Epimastigotes*<br>(Franco *et al.*, 2015[37])<br>The method used was same as summarized above for the study by Moreno *et al.*, 2010[35] | Silvio X 10 cl1<br>TcI | 26.1 µM<br>6.79 µg/mL |
| | Colombiana | 40.5 µM |

| Parasite stage (Reference) and experimental design | Strain (DTU) | $IC_{50}$ |
|---|---|---|
| | TcI | 10.54 µg/mL |
| | YuYu<br>TcI | 40.5 µM<br>10.54 µg/mL |
| | VL10<br>TCII | 30.4 µM<br>7.91 µg/mL |
| | BE-62<br>TCII | 14.6 µM<br>3.80 µg/mL |
| | 893<br>TCIII | 74.9 µM<br>19.49 µg/mL |
| | SO3 cl5<br>TcV | 10.6 µM<br>2.76 µg/mL |
| | CL Brenner<br>TCVI | 13.2µM<br>3.44 µg/mL |
| | 793<br>Tcbat | 16.6 µM<br>4.32 µg/mL |

*Nonresponsive strains and **Responsive strains based on based on long-term treatment of mouse (CD1 mice) infections with nifurtimox and BZN.
DTU-discrete typing units. BE-Berenice; Vero cells- African Green monkey cells

## Appendix-2

Summary of nonclinical studies supporting activity of benznidazole in animals infected with *T. cruzi*

| ***T. cruzi* strains/clones Reference** | **Study summary** |
|---|---|
| **Mice** | |
| **Acute infection** | |
| Y strain<br><br>Polak and Richle, 1978[28] | Albino mice were infected IP with approximately$10^5$ blood stage. Mice developed increasing parasitemia from Day 4 and died within Days10-14 PI. A single dose of 1000 mg/kg BZN was administered on Days 7 or 8 PI. Blood was collected 0-96 hours after treatment and number of trypanosomes counted by phase contrast microscopy. A decrease in parasite count was observed ≥6 hours post-treatment. All treated mice were negative by 12 hours post-treatment. |
| 30 strains<br>3 - Type I (Y-Brazil; Peruvian-Peru; Buenos Aires-Argentina),<br><br>14 - Type II (from Mambai, Sao Felipe, and San Luis)<br><br>13 - Type III [from Montalvania, La Pampa, Santa Cruz la Sierra, and Columbia (Colombiana)]<br><br>Andrade *et al*., 1985[13] | Mice infected IP with $2x10^5$ blood stage of different strains maintained by serial passage in mice; mice were treated orally with BZN for 90 days. The authors state that Type I strain is characterized by a rapid course of infection in mice, high levels of parasitemia and mortality around the 9th and 10th day of infection; there is predominance of slender forms and macrophage-tropism during the acute phase of the infection. Type II are characterized by increasing parasitemia from the 12th to the 20th day of infection, a low rate of mortality, a predominance of broad forms of the parasites, and myocardial tropism. Type III strains show a slow development of parasitemia that reaches a high level 20-30 days after inoculation, a low mortality, and predominance of parasitism in skeletal muscles. The activity was measured by direct microscopic examination, xenodiagnosis, inoculation of blood in new-born mice, and hemoculture and serological test (IFA). Cure rates were based on parasite clearance on Days 30-90 post-treatment.<br><br>The cure rates in mice infected with the Type I stains were between 50% and 100%; Type II strains between 12.5% and 100%; and Type III strains 0%-20%. Type I strains displayed high susceptibility, Type II strains medium to high susceptibility, and Type III strains were highly resistant.<br><br>About 76% of the parasitologically negative mice remained seropositive at a dilution of 1:10 to 1:80. |
| 5 strains: Sonya clone*, Colombiana*, Tulahuen**, Y** and Peru**.<br><br>Neal and van Bueren (1988)[32] | CD-1 mice were infected SC with $10^5$ trypomastigotes of the 5 strains of *T. cruzi*. Oral treatment with BZN (30-120 mg/kg for 28-30 days) was initiated about 7 days PI, when parasitemia occurred. Mice were followed for parasitemia by microscopic examination of blood collected at the end of dosing and 28 days later. If all samples were negative, blood cultures were performed in triplicate. Mice were necropsied and tissues (skeletal muscle from the back of the thigh, heart, bladder, kidney, liver, esophagus, ileum, colon and spleen) collected were processed for histological examination of pseudocysts.<br><br>In all untreated mice infected with either of the strain, high parasitemia was reported leading to death. The mean time to death was 13.8 days for Tulahuen and Y strains; 14.8 days for strain Peru; 17.9 days for Sonya clone; and 21.1 days for Colombiana strain.<br><br>Treatment with BZN was effective in curing a majority of the mice infected with the responsive strains (Tulahuen, Y and Peru strains). However, all mice infected with the Colombiana strain were hemoculture positive; 4/9 mice infected with the Sonya clone and treated with the highest dose (120 mg/kg) of BZN were culture positive. Survival of treated mice at different time points was not shown. |
| 18 strains from the Brazilian States of Rio Grande do Sul and Minas Gerais belonging to zymodemes Z1, Z2 and ZB<br><br>Murta *et al*., 1998 | Albino mice were infected IP with $10^4$ blood stream forms of the different *T. cruzi* strains. If the parasitemia was low in normal mice, the mice were immunosuppressed by irradiation. At the time of peak parasitemia, mice were treated with a single dose of BZN (500 mg/kg) and reduction in parasitemia measured 3 and 6 hours post-treatment. The strains were characterized as (i) susceptible when experimentally infected mice presented a 80–100% reduction in parasitemia, (ii) moderately resistant when they presented a 20–80% of reduction in parasitemia, and (iii) resistant when the percent reduction was 0–20% at 6 hours post-treatment.<br><br>The parasite reduction varied from 15% to 100%; one strain (MAGUI- zymodeme 1) was characterized as resistant with parasite reduction of 15%; the susceptibility against the 4 strains [TS 478-(zymodeme 1), RS-5, RS-6, and 229A (zymodeme 2)] was between 64 and 72%; the remaining 13 strains were susceptible and were zymodeme 1, 2, or B). Independent of the state of |

| ***T. cruzi* strains/clones Reference** | **Study summary** |
|---|---|
| | origin, strains from zymodeme B (n=10) were all susceptible whereas strains from zymodemes 1 and 2 showed a range of sensitivities. |
| CL**, Y#, Colombiana*, SC-28*, and VL10*<br><br>Molina *et al*., 2000[40] | Swiss albino mice were infected IP with $10^4$ trypomastigotes of the different strains. Testing was performed in both immunocompetent and immunosuppressed mice; immunosuppression was induced by treatment with two doses of cyclophosphamide (IP, 50 mg/kg of body weight/Day, 2 and 1 day before infection). Oral treatment with BZN (100 mg/kg/day) was initiated on Day 4 PI for 20 or 28 days. Mice that were treated for 28 days were administered a second course of treatment for 15 days after a 7-day rest period. Mice were followed until Day 113 for parasitological (hemoculture and xenodiagnosis) and serological response (IFA); labeled parasites were analyzed by cytofluorometry. Mice were considered cured if all 3 tests were negative.<br><br>In mice treated for 20 days, compared to the control group, treatment with BZN was effective in improving survival of mice infected with the CL, Y, or Colombiana strain. However, the survival was maximal (100%) for the immunocompetent treated mice infected with the CL strain and all mice were parasitologically cured. Among the mice infected with the Y strain, 90% survived and 50% were parasitologically cured. About 80% of mice infected with the Colombiana strain survived but none were parasitologically cured. In the immunosuppressed mice, compared to immunocompetent mice, survival rate and survival time were lower.<br><br>In immunocompetent mice treated for 28 days, followed by a 7-day rest and another 15 days of treatment, for a total of 43 doses, BZN was effective in improving survival in 90% and 80% of mice infected with the CL and Y strains; the survival rates were 60%, 70% and 70% for mice infected with the Colombiana, SC-28, and VL-10 strains. All the BZN treated mice infected with the CL strain were cured.<br><br>Antibody response in cured and uncured mice was not shown. |
| Colombiana Type I parenteral strain and Col A strain and Col B collected after 8 and 17 years of infection.<br><br>BE-78 parenteral strain and isolates collected after 7 (BE-78 B and C) and 2 (BE-78 D strain) years of infection.<br><br>Veloso *et al*., 2001[41] | Swiss outbred mice were infected IP with 5 x $10^3$ blood trypomastigotes isolated from dogs infected with different strains or stocks of *T. cruzi* [parenteral strains: 8 (Col A strain) and 17 (Col B strain) years of infection with the Colombiana Type I (resistant to BZN) strain and BE-78 (BE-78) Type 2 strain after 7 (BE-78 B and C) and 2 (BE-78 D strain) years of infection]. After the detection of parasitemia (approximately 4 days PI), the mice were treated with BZN (100 mg/kg for 20 days). Mice were considered cured when both parasitological (fresh blood examination, hemoculture and PCR) and serological (ELISA) tests were negative. PCR was performed only of samples from animals that were hemoculture negative.<br><br>Mice infected with Col strain and isolates Col A and Col B did not respond to treatment with BZN. On other hand, mice infected with BE-78 strain showed 100% of cure after treatment, whereas mice infected with their isolates BE-78B, C and D) displayed different spectra of susceptibility to BZN, with 100%, 50% and 70% cure rates, respectively. |
| Colombiana strain prototype of Biodeme Type III and 7 clones (Cl-Col-C1, C2, C3, C4, C5, C6 and C7)<br><br>Camandaroba *et al*., 2003[42] | Swiss outbred mice were infected (route of infection not specified) with 5 x $10^4$ to 1 x $10^5$ trypomastigotes of either the Colombiana strain or 7 clones. Peak mortality occurred at Day 30 PI; mortality rate varied and was lowest in mice infected with the C2 clone. All the mice were parasitemic (either by direct blood examination, subinoculation into newborn mice or hemoculture). Treatment with BZN (10 mg/kg/day by gavage) was initiated on Day 20 PI for 90 days. Direct parasitological examination of blood was performed at the end of treatment. Hemoculture, subinoculation into experimentally naïve suckling mice as well as serological (IFA using culture forms of *T. cruzi*) and molecular (PCR) testing were performed on animals that were negative by direct examination of the parasite.<br><br>The results show that treatment with BZN improved survival time of mice infected with the Colombiana strain or the clones. The survival rate against different clones varied; the mortality was higher in C1(80%), C4 (75%) and C6 (60%) infected treated mice compared to mice infected with other strains (parenteral-20%; C2 and C3-10%; C4-25%; C5-50%; C7-30%). About 73% of the untreated infected mice, irrespective of the strain, died. |

| ***T. cruzi* strains/clones Reference** | **Study summary** |
|---|---|
| | Cure rates based on parasitological findings varied. Based on positivity in at least one parasitological (direct blood examination, subinoculation into newborn mice or hemoculture ) or serological test, on Day 30, all the treated mice infected with either Colombiana strain or any of the clones except C3 failed treatment; 17% of the treated mice infected with C3 clone were cured. PCR did not appear to be very sensitive compared to other parasitological tests; 29.2% of blood samples from treated mice were positive by PCR versus 40% of the controls (infected, not treated).<br><br>Antibody titers by IFA were performed at 30 days after the end of treatment and antibody positive findings were based on serum dilutions $\geq$ 1:20; 69% of the mice infected with the C3 clone were antibody positive whereas 89% of the mice infected with the parenteral strain and 100% of the mice infected with the other strains were positive. Data to evaluate correlation between survival, parasitological and serological findings were not available in the publication. |
| Colombiana* CL**, and Y[#]<br><br>Urbina *et al*., 2003[43] | NMRIIVIC albino mice were infected with $10^4$ trypomastigotes, IP, of different *T. cruzi* strains. Treatment with BZN (100 mg/kg/day for 20 days by gavage) was initiated 4 days PI. Mice were followed until Day 60. A majority (75 – 82%) of the untreated mice died during the period of observation; all the surviving animals were parasitemic. All the treated mice survived the period of observation. All the treated mice infected with the CL strain were aparasitemic whereas 25% and 67% of the treated mice infected with the Y and Colombiana strains, respectively, remained parasitemic. |
| Y<br><br>Ferraz *et al*., 2007[44] | C57BL/6 WT and IL-12-12 p40 or IFN-γ knockout (KO) mice were infected IP, with $5x10^3$ trypomastigote and oral treatment with BZN (100 mg/kg/day for 20 days) was initiated on Day 4 PI. Mice were followed for parasitemia, by microscopy, up to 60 days PI. If the mice became aparasitemic on Day 60 PI, hemoculture was performed by incubating cultures for 30 to 60 days at 28°C.<br><br>Untreated WT mice became parasitemic at Day 4 PI that peaked at Day 9 and was undetectable by Day 16 PI; circulating parasites appeared intermittently up to Day 60 PI. None of the untreated mice were cured by direct examination or hemoculture. A majority (91%) of the mice died by Day 60 PI with mean survival time of 21.2 days Parasites were not detected at any of the time points in the treated mice and all mice survived the period of observation. Mean survival time increased in treated mice (>60 days) compared to the WT mice (21.2 days).<br><br>IFN-γ-KO mice were more susceptible to *T. cruzi* infection and less responsive to BZN treatment compared to the WT mice. Peak parasitemia levels for the untreated mice were 8-fold higher and the survival time shorter (13.5 days) compared to 21.2 days in WT animals. Treatment with BZN was effective in decreasing parasitemia (subpatent); however, reactivation occurred 2 days after discontinuation of treatment, parasitemia peaked on Day 11 and all mice died by Day 48 PI. Mean survival time increased in treated (42.1 days) and WT (>60 days) mice compared to untreated mice.<br><br>IL-12 KO mice were more susceptible to *T. cruzi* infection and parasitemia peaked at Day 9; peak parasitemia was slightly higher than in WT mice and less than IFN-γ-KO mice. Treatment with BZN was effective in reducing parasitemia to subpatent level; however, low levels of circulating parasites appeared 14 days after the end of treatment with parasites persisting intermittently throughout the rest of the observation period. Mean survival time increased in treated mice (59.6 days) compared to the WT mice (12.3 days). |
| CL - strain expressing tdTomato protein or luciferase<br><br>Canavaci *et al*., 2010[34] | BALB/c mice were infected with $2.5x10^5$ of tdTomato or $1x10^5$ of luciferase expressing trypomastigotes into the footpad; oral treatment with BZN (100 mg/kg) was administered between Days 6 and 11 and Days 4 and 10, respectively. The parasite load at the site of infection was measured by the fluorescence and bioluminescent intensity (photons/cm$^2$/sec). A reduction in the intensity of infection was observed within one day of treatment with BZN compared to the untreated control mice thereby suggesting rapid clearance.<br><br>C57BL/6 mice were infected with 1000 trypomastigotes of the WT CL strain; oral treatment with BZN (100 mg/kg) was initiated on Day 15 for 40 days. All mice were parasitemic by Day 14 PI. In untreated mice, the parasitemia peaked at Day 21 PI; parasites were undetectable by Day 35 PI. In BZN treated mice, parasites were undetectable by Day 21. An immunosuppressive agent, cyclophosphamide, was administered on Days 105, 108, 111, 113, and 117 PI. Unlike the untreated mice, no parasites were detected in blood from mice treated with BZN. |

| ***T. cruzi* strains/clones Reference** | **Study summary** |
|---|---|
| Y<br><br>Olivieri *et al*., 2010[45] | Outbred Swiss albino mice were infected, IP, with $10^4$ trypomastigotes and treatment with BZN (100 mg/kg/day) by gavage initiated on Day 4 PI for 20 days. Parasitemia was measured by light microscopy and survival measured until 150 days PI. Hemoculture was performed on Days 30 and 54 PI. Heart and spleen from a subgroup of animals were collected on 8, 15, 35 and 150 days PI; heart tissue was processed for histopathological evaluation and immunofluorescence staining with anti-fibronectin or anti-laminin antibodies and 4´,6-diamidino-2-phenylindole (DAPI). Spleen was weighed and cell suspension prepared for phenotyping. Serum samples were collected for measuring creatine kinase (CK) isoform MB (a surrogate marker of cardiac damage) as well as anti-*T. cruzi* IgG antibody titers against the crude extract of the Y strain by ELISA. A serum sample was considered positive when its optical density value was higher than the mean of three seronegative healthy mice plus 3 standard deviations.<br><br>The peak parasitemia occurred at 7–8 days PI and all untreated mice died by Day 35 PI. All the mice treated with BZN survived until Day 150 PI. Hemoculture was positive in 4/8 mice on Day 54. The authors state that anti-*T. cruzi* IgG antibodies increased with the evolution of infection, starting at Day28; in BZN treated mice, the antibody titers were lower and comparable to uninfected controls (data not shown).<br><br>By histopathology, presence of parasites and inflammatory infiltrates was observed on Day 8 PI that increased during the progression of infection; at Day 15 PI, intense inflammatory infiltrates with diffuse mononuclear foci, abundant parasite nests and necrosis were observed. In BZN treated mice, no inflammation or parasites were observed between Days 11 and 150 PI. By immunofluorescence staining, an increase of fibronectin deposition and the number of DAPI-stained nuclei owing to the presence of inflammatory infiltrates at 15 days PI was reported; in BZN treated mice, such an increase was prevented and was similar to heart tissue from uninfected control mice.<br><br>The CK-MB increased rapidly in infected mice. In BZN treated mice, CK-MB levels decreased compared to infected untreated group but were higher than the uninfected control group.<br><br>BZN was effective in reducing splenomegaly compared to infected untreated mice; however, the spleen was enlarged compared to the uninfected control mice. In BZN treated mice, splenocyte number was reduced on Day 8 PI but increased on Day 15 PI. There was an increase in the number of $CD4^+$ and $CD8^+$ cells expressing CD69 and CD44. By Day 150 PI, all mice recovered from spleen enlargement and numbers of different T-cell subsets were similar to control uninfected mice. |
| 23 strains: TcI (n=6), TcII (n=4) and TcIV (n=13)<br><br>Teston *et al*., 2013[46] | Swiss mice (n=20/group) were infected IP with $10^4$ trypomastigotes of 19 of the 23 strains (for 4 strains 200 trypomastigotes were used), belonging to three different DTUs of *T. cruzi* obtained from humans in the acute phase of Chagas disease, triatomines and marsupials in the state of Amazonas and from chronic patients and triatomines in the state of Paraná, Brazil. Treatment with BZN was initiated on Day 5 PI for 20 days. Parasitemia was measured on Day 3 PI; some mice were treated without conformation of parasitemia e.g., TcI strain from Amazonas - parasitemia was subpatent. The authors state that these strains show high infectivity to mice. Mice with negative fresh blood examination, hemoculture, PCR and ELISA [using alkaline antigen of the Y (TcII) strain] results after the treatment ended were considered cured; those with at least one positive result in any test were considered not cured.<br><br>Despite the variability in the progression of parasitemia and time to peak parasitemia, treatment with BZN was effective in decreasing parasitemia in mice infected with either of the strains of all the 3 DTUs. The cure rates varied between 27 and 100%. The cure rates for TcI strains from Amazonas were significantly higher (P = 0.028) than for the TcI strains from Paraná. The % mice that were parasitemic by direct examination, hemoculture, PCR, or by ELISA were decreased in BZN treated mice compared to the untreated control group. The % of mice that were antibody positive increased from Month 3 to 6; however the % antibody positive mice in treated group were lower than in the untreated group. In the untreated group an increase in the % antibody positive was evident in mice infected with the TcI strains but not in mice infected with the TcII or TcIV strains; this could be due to the fact that a majority of the mice infected with TcII or TcIV strains were antibody positive by Month 3. |
| CL, Brazil, | C57BL/6 ($Ly5.2^+$) mice were infected IP with $10^3$ trypomastigotes of different strains of *T. cruzi*. |

| ***T. cruzi* strains/clones Reference** | **Study summary** |
|---|---|
| Montalbania, Colombiana strains<br><br>Bustamante *et al*., 2014[47] | Treatment with BZN (100 mg/kg), by gavage, was initiated on Day 15 for 40 days. The infected BZN treated mice were immunosuppressed with cyclophosphamide (200 mg/kg/day) IP at 2–3 day intervals for a total of 5 doses. Hemoculture was performed for immunosuppressed mice every week for 2 months. On Day 120, no parasites were detected by PCR and there was no histological evidence of infection in the skeletal muscles of treated mice compared to untreated mice. There was a decrease in the frequency of *T. cruzi*–specific $CD8^+$ T cells as well as an altered T-cell memory phenotype in BZN treated mice that were cured compared to untreated mice or treated mice that were not cured. The strain of *T. cruzi* used in this experiment was not specified.<br><br>In mice infected with the Colombiana strain and treatment initiated on Day 15 PI for 40 days, a rapid reduction in parasitemia to undetectable level was reported. However, one-third of the mice showed a re-occurrence of parasites in blood at Day 150 PI and a further exacerbation after cyclophosphamide immunosuppression, whereas the remaining treated mice appeared parasite-free after immunosuppression. Cured mice showed lower frequency of *T. cruzi*–specific $CD8^+$ T cells as well as an altered T-cell memory phenotype. Similar observations were made when treatment was extended to 60 days in place of 40 days.<br><br>Similar observations were made in mice infected with the Montalbania strain.<br><br>In another experiment, mice were infected with Brazil strain (BZN sensitive) and intermittent treatment (for 5 or 10 days with a 5 day interval) with BZN initiated on Day 15 for 40 or 60 days; mice were immunosuppressed with cyclophosphamide between Days 120 and 135. The cure rates on Day 135 were between 45% and 75% for mice treated with 9 doses of BZN at 5-day intervals or a combination of initial daily treatments (5 or 10 days) followed by treatment at 5-day intervals for a total of 40 days. Extending the intermittent treatment period to 60 days increased the cure rate to levels approaching that of daily 40-day treatment courses (95%–100%). |
| CL Brener<br><br>Francisco *et al*., 2015[48] | BALB/c mice were infected IP with $10^3$ trypomastigotes of a bioluminescent reporter clone derived from the genome reference strain CL Brener that had been grown *in vitro* or passaged in CB17 SCID mice. On Day 14 PI, infected mice were treated with BZN (100 mg/kg/day) for 20 days. To facilitate the detection of residual infection after cessation of BZN treatment, BALB/c mice were immunosuppressed, in some experiments, with cyclophosphamide (200 mg/kg, IP) at 3- to 4-day intervals, for a maximum of three doses. D-luciferin was injected for efficacy assessment by *in vivo* and *ex vivo* imaging; for *ex vivo* imaging, mice were sacrificed by exsanguination and different organs and tissues were processed for imaging.<br><br>By *in vivo* imaging, peak parasitemia occurred after 14 days followed by a reduction in parasite load during progress to the chronic stage at 40 to 50 days PI. There was a rapid reduction in bioluminescence to undetectable level in BZN treated animals; also, no signs of infection were observed in immunosuppressed mice or immunocompetent mice until Day 74. No luminescence was detected in the organs from animals treated with BZN. |
| **Chronic infection** | |
| CL**, Y[#], Colombiana*, SC-28*, and VL10*<br><br>Molina *et al*., 2000[40] | Swiss albino mice were infected IP with 30 trypomastigotes of the different strains; this led to a more controlled infection and higher survival levels. After 20 days, surviving mice had developed a chronic latent infection with no circulating parasites; mice were treated orally with BZN for 20 days and animals followed until Day 191 PI for parasitological and serological (by IFA) response. Like for the acute infection model, mice were considered cured if all 3 tests were negative.<br><br>BZN was effective in improving survival of 40% of the mice infected with either the CL, Y, or Colombiana strain; however, the difference was statistically significant only for mice infected with the Colombiana strain. No parasites were detected in any of the infected mice, treated or untreated.<br><br>Antibody response in cured and uncured mice was not shown. |
| Colombiana<br><br>Garcia *et al*., 2005[49] | BALB/c mice were infected IP with 100 trypomastigote forms of the Colombiana strain of *T. cruzi*. On Day 45 PI, daily oral treatment with BZN (100 mg/kg/) was administered; this was followed by weekly administration for 8 months. Parasitemia were performed at different time intervals. ECGs were performed at Month 10. At necropsy, heart tissues were collected and processed for histological examination and detection of parasites by IFA. Serological testing (ELISA) was performed to detect the presence of antibodies against *T. cruzi* epimastigotes, recombinant trans- |

| ***T. cruzi* strains/clones Reference** | **Study summary** |
|---|---|
| | sialidase (TS), $P_2\beta$, $M_2$-muscarinic and β1-adrenergic cardiac receptors. The authors state that the TS is an enzyme abundant on the *T. cruzi* parasite surface and an immunodominant antigen recognized by sera from patients with Chagas' disease.<br><br>A reduction in the detectable parasite foci (3-fold) as well as inflammatory cells (2-fold) was observed in the hearts of mice treated with BZN compared to the untreated mice at Month 10 PI. However, there was no significant differences in the fibrotic areas of the hearts between untreated and BZN-treated mice. Although both untreated and treated infected mice had significant alterations in their ECGs compared to those of the healthy mice, about 20% of the BZN treated mice had cardiac conduction disturbances including intraventricular conduction disturbances, atrioventricular blocks, and extrasystoles than untreated infected mice.<br><br>Antibody titers against *T. cruzi* antigens, epimastigote extract, TS, $P_2\beta$, as well as antibodies against the peptides of the second extracellular loops of β1-adrenergic and $M_2$-muscarinic cardiac receptors were lower in the sera from BZN-treated mice compared to the sera from untreated mice. |
| CL, Brazil, Montalbania, and Colombiana<br><br>Bustamante *et al*., 2014[47] | C57BL/6 ($Ly5.2^+$) mice were infected IP with $10^3$ trypomastigotes of different strain of *T. cruzi*. Treatment with BZN (100 mg/kg) by gavage was initiated on Day 120 for 40 days. On Day 240, the infected BZN treated mice were immunosuppressed with cyclophosphamide (200 mg/kg/day, IP) at 2–3 day intervals for a total of 4 doses. BZN treatment in the chronic phase of infection with the Colombiana strain (120–160 days PI) suppressed parasitemias but failed to produce cure.<br><br>In another experiment, mice were infected with Brazil strain (BZN sensitive) and intermittent treatment with 13 doses of BZN was initiated on Day 130 over a course of 60 days; mice were immunosuppressed with cyclophosphamide between Days 233 and 242. On Day 245, all mice were cured. |
| CL Brener<br><br>Francisco *et al*., 2015[48] | BALB/c mice were infected IP with $10^3$ trypomastigotes of a bioluminescent reporter clone derived from the genome reference strain CL Brener and the experimental design was same as summarized above for the acute model except that treatment was initiated on Day 74 for 20 days. Like for the acute infection model, there was a rapid reduction in bioluminescence to undetectable level within 5 days of treatment with BZN; also, no signs of infection were observed in immunosuppressed mice or immunocompetent mice until Day 74. No luminescence was detected in the organs from animals treated with BZN. Spleen weight in infected BZN treated mice was lower than the infected untreated mice and same as the non-infected mice.<br><br>In another experiment, BZN (100 mg/kg) treatment was initiated on Day 103 PI for 5 or 10 days. In BZN treated mice, bioluminescence fell below the level of detection by the completion of treatment as assessed by either *in vivo* or *ex vivo* imaging; no relapse was observed after immunosuppression. |
| **Rabbits** | |
| Ernestina<br><br>Teixeira *et al*., 1990a[52] | New Zealand white rabbits were infected SC with $10^6$ trypomastigotes/kg body weight. Parasitemia was monitored by xenodiagnosis and repeated every 15 days for 6 months and every month thereafter; xenodiagnosis was performed using 20 first instar nymphs of the reduviid bug (*Dipetalogaster maximus*). A positive diagnostic test was indicated by the presence of the parasite in the feces of bug fed on the rabbit's blood; feces were examined 30 and 60 days after exposure. Treatment with BZN (8 mg/kg/day for 60 days) was administered IP. The time of initiation of treatment was not specified. Complete necropsies were performed on each rabbit and all organs and tissues were processed for histopathological evaluations.<br><br>Parasitemia in infected rabbits lasted for 5 months, after which time the parasites disappeared spontaneously. Compared to untreated rabbits, treatment with BZN reduced the duration of parasitemia by 75%. Mycocarditis and lymphomas were reported in both BZN treated and untreated infected rabbits. Lymphomas were also reported in non-infected rabbits administered BZN. |

| ***T. cruzi* strains/clones Reference** | **Study summary** |
|---|---|
| Ernestina<br><br>Teixeira *et al*., 1990b[53] | The effect of treatment with BZN was measured during the **acute phase** (BZN treatment initiated 2 months PI) as well as during the **chronic phase** (BZN treatment initiated 6 months PI). Otherwise, the experimental design was same as summarized above, except that immune response was measured by (a) direct agglutination of trypsin treated and formalin-killed *T. cruzi* culture forms, (b) hemolytic antibodies were measured against a heterologous antigen (the sheep RBCs); for this rabbits were immunized with sheep RBC stromata, (c) delayed-type skin reaction against *T. cruzi* soluble antigen (TCSA). The TCSA was prepared from the parasite forms (92 ± 8% epimastigotes and 8 ± 4% trypomastigotes) grown in UT medium. The TCSA contained in the supernatants was used for skin tests. The intradermal injection of 50 µg protein in 100 µL TCSA resulted in local indurated inflammatory reaction, the diameter of which was registered after 48 hours.<br><br>The survival time of untreated *T. cruzi*-infected rabbits was 765 ± 639 days; infected rabbits treated with BZN during the acute and chronic phase of infection, survived for 525±714 days and 392 ± 571 days, respectively. The peak parasitemia in all rabbits was reached at Day 45 of infection; at that time, ~35% of the *D. maximus* fed on infected rabbits became infected with *T. cruzi*. On Days 75 and 90, parasitemia was detected in 25% and 27% of bugs, respectively fed on untreated rabbits; 5% and 0% of the bugs fed on BZN treated rabbits were positive on Days 75 and 95, respectively. Persistence of parasites, in untreated rabbits, was observed for 5 ½ months. Animals were aparasitemic after that.<br><br>Mycocarditis developed in all infected animals, treated and untreated.<br><br>Agglutinating antibodies against the parasite envelope were detected 15 days PI; the antibody titers increased by Month 2 in BZN treated (acute or chronic phase) and untreated rabbits; the antibody titers remained high until Month 20 PI.<br><br>Antibodies against the sheep RBC stromata were not altered by treatment with BZN. However, suppression of the delayed-type skin reaction against *T. cruzi* soluble antigen as well as PPD was reported during the BZN treatment phase (both acute and chronic); suppression of delayed type cellular response was reversible within 10 days of discontinuation of BZN treatment. |
| **Dogs** | |
| **Acute infection** | |
| Colombiana, (Tc I), Y (TcII), and/or BE-78 (TcII)<br><br>Guedes *et al*., 2002[54] | Mongrel dogs were infected IP with $2x10^3$ trypomastigotes/kg of Colombiana, Y or BE-78 strain and BZN (7 mg/kg bid) treatment was initiated immediately after dogs became parasitemic (12 to 22 days PI) for 45 days. Parasitemia was followed at different time intervals by 4 methods; fresh blood examination, hemoculture, PCR assay, and complement mediated lysis (CoML; using trypomastigotes of the Y strain; the results were considered positive when lysis was greater than 20%) and ELISA using antigen from the epimastigotes and trypomastigotes of the Y strain-for ELISA, the cut-off was determined using the absorbance mean of 10 uninfected animals plus two standard deviations. The animals were considered cured when all the tests were negative. All the dogs survived the period of observation. All untreated control animals were positive for hemoculture and PCR. Parasitemia was suppressed within 3 days post-treatment in all treated dogs. However, one dog infected with the Colombiana strain remained hemoculture positive and all the 4 dogs remained PCR positive. One dog infected with the BE-78 strain became hemoculture negative at Month 6 post-treatment; one dog remained PCR positive.<br><br>The antibody titers in infected dogs treated with BZN were lower than in untreated dogs against both epimastigote and trypomastigote antigens of the Y strain; the decrease was more in dogs that were parasitologically cured. Based on hemoculture, PCR and serologic (ELISA-epimastigote and CoML) findings, 68.75% (11/16) of the treated animals were cured. All the untreated dogs were antibody positive by the CoML assay. Of the treated dogs, 5 dogs (1 infected with the BE-78 and 4 with the Colombiana strain) were antibody positive at Month 6 post-treatment.<br><br>Based on hemoculture, PCR and serologic (ELISA-epimastigote and CoML) findings, 68.75% (11/16) of the treated animals were cured |
| Y (TcII) and BE-78 (TcII) strains | Mongrel dogs were infected with $2x10^3$ trypomastigotes/kg of Y or BE-78 strains. BZN (7 mg/kg bid) treatment was initiated immediately after dogs became parasitemic (12 to 22 days PI) for 60 days. Parasitemia was followed at different time intervals by 4 methods [fresh blood examination, |

| ***T. cruzi* strains/clones Reference** | **Study summary** |
|---|---|
| de Figueiredo Diniz *et al*., 2010[55] | hemoculture, PCR assay, and ELISA using alkaline extracted antigen from the Y strain]. The control (untreated) dogs infected with the Y strain showed the highest parasitemia levels; the peak parasitemia was 25,000 trypomastigotes/0.1 mL of blood and the patent period was 12 to 13 days; in dogs infected with the BE-78 strain, the peak parasitemia was 15,000 trypomastigotes/0.1 mL of blood and a patent period was 10 to 19 days. A majority of the dogs survived the period of observation.<br><br>BZN was effective in suppressing parasitemia (culture and PCR) in dogs infected with either the Y or BE-78 strains and all the animals survived.<br><br>Antibody titers by ELISA increased until about the 90$^{th}$ day of infection and stabilized afterwards until the end of the experiment. There was a difference in antibody response in animals infected with the Y or BE-78 strain of *T. cruzi*. Treatment with BZN reduced IgG, IgG1 and IgG2 levels during the 6 month observation period.<br><br>At Month 6 after the end of treatment, histopathological evaluation of the heart tissue was performed. BZN was effective in reducing the inflammatory and fibrotic cardiac lesions, in both Y-infected dogs; also, BZN was effective in reducing IFN-γ expression and increasing IL-10 expression in the heart tissue compared to the untreated control group. |
| **Chronic infection** | |
| BE-78 (TcII) Guedes *et al*., 2002[54] | Experimental design same as above for the acute infection model except that dogs were infected with the BE-78 strain and the treatment was initiated on Day 100 PI. None of the dogs were parasitologically cured.<br><br>By epimastigote ELISA, a decrease of antibody titers was greater in treated dogs compared to the untreated dogs. However, by the trypomastigote ELISA, such changes in the antibody response were not observed. The CoML antibodies were present in all animals, treated and untreated, at Month 9 post-treatment; at Month 24, CoML were reduced in dogs that were parasitologically cured compared to the treated dogs that were not cured.<br><br>By hemoculture, PCR and serological tests, 3 of the 8 dogs were considered cured. |
| BE-78 (TcII)<br><br>Santos *et al*., 2012[56] and Santos *et al*., 2016[57] | Mongrel dogs infected IP with 4x10$^3$ trypomastigotes/kg body weight and treatment with BZN initiated 4 months PI for 60 days. The effect on tissue parasitism and its correlation with ameliorating cardiac systolic function was evaluated by echo Doppler cardiographic exams performed before infection and at Months 6 and 18 PI. Parasitic load was measured by PCR (kinetoplast DNA) in blood and heart tissue.<br><br>The results show that BZN treatment was effective in reducing parasite load in blood and heart at Month 1 post-treatment compared to untreated animals; this was associated with an improvement in systolic heart function. However, at Month 12 post-treatment, there was an increase in parasitic load in the blood and heart tissue as well as cardiac dysfunction.<br>Cytokines (IL-10 and TNF-α) levels were measured by ELISA *ex vivo* in the supernatants of the PBMC isolated at Months 1 and 12 after treatment and cultured with trypomastigote antigen. The results showed an increase in IL-10 levels and no change in TNF-α levels at Month 1 in both infected untreated and BZN treated dogs compared to uninfected dogs. However, at Month 12, the IL-10 levels decreased whereas the TNF-α levels increased compared to uninfected controls. The elevation of TNF-α synthesis and the reduction in IL-10 synthesis by PBMCs were not altered by BZN treatment. Such an in immunological imbalance may contribute to the progression of cardiomyopathy disease.<br><br>The temporary suppression of the *T. cruzi* infection induced by BZN treatment was efficient in reducing systolic cardiac function alterations, but not in preventing the development of cardiomyopathy. |
| *represent non responsive and **represent responsive (for details see Appendix-1) and #partially responsive strains PI-post-infection; BZN-benznidazole; IP-intraperitoneal; SC-subcutaneous; WT-wild type; PBMC- peripheral blood mononuclear cells | |

# Appendix-3

## Summary of serological and molecular assays used in clinical studies

Table A: Study 1 (Sosa-Estani *et al*., 1998[1]) - Summary of methods in the Lab standardization manual and differences with the information available from cross-referenced publication.

| Test** | Based on Lab standardization report | Differences in the method from that in the cross-referenced publications |
|---|---|---|
| IHA | • Mixing different dilutions of patient's serum and red blood cells (sheep or chicken, stabilized with formalin, sensitized with *T. cruzi* cytoplasmic and membrane antigens).<br>• Observation - RBCs are agglutinating and readout.<br>• Cut-off: ≥1:32 dilution as positive. | Cerisola *et al*., 1971[69]*<br>• Human blood group O RBCs were used. Parasite strain and stage of the parasite used as antigen not specified.<br>• Sensitivity 95%; Specificity 98%; non-specificity of 2% due to cross-reactivity with African trypanosome 4.6%; none with *Leishmania*, *Toxoplasma*, amebiasis, syphilis. |
| IFA | • Preparation of microscopic slides with epimastigotes of the Tulahuen strain of *T. cruzi*.<br>• Incubated with serum (heat-inactivated) and controls.<br>• Detection with anti-human-IgG labelled with FITC, diluted in Evans Blue.<br>• Readout fluorescence in microscope.<br>• Cut-off: ≥1:32 dilution as positive. | Alvarez *et al*., 1968[71]*<br>• Different strains, fixed in formalin from Argentina used. Unclear if Tulahuen strain. Trypanosomes cultured in glucose blood agar used as antigen,<br>• Anti-human globulin conjugated with FITC.<br>• Sensitivity higher than IHA. Cross-reactions with mucosal leishmaniasis[!] |
| **ELISA** | | |
| *Conventional*<br><br>Whole cultured parasite extract (probably epimastigotes) | • Soluble antigen coated on microtiter plates; strain of parasite not specified.<br>• Incubated with plasma samples and control samples.<br>• Detection with conjugate containing a peroxidase-labelled rabbit-anti-human IgG antibody followed by addition of peroxidase substrate reaction and readout (OD) in a spectrophotometer at 490 nm.<br>• Cut-off - OD >0.200 as positive. | Voller *et al*., 1975[72]<br>• Peru strain of *T. cruzi.* Stage not specified.<br>• Rabbit-anti-human immunoglobulin labelled with alkaline phosphatase and alkaline phosphatase substrate used. OD at 400 nm.<br>• All the 26 patients with positive by xenodiagnosis were positive by ELISA.<br>• Cut-off: >0.4 OD as positive |
| *Non-conventional – F29*<br><br>[Flagellar calcium-binding protein (F29) as antigen from epimastigotes] | • F29 antigen (5 µg/mL; 50 µL/well).<br>• Similar steps to that summarized above for conventional ELISA except that the OD was determined at 492 nm.<br>• Cut-off - OD ≥0.170 as positive.<br>The cut-off was determined based on 50 sera from healthy individuals: Average OD ± 3 SD. | •Porcel *et al*., 1996[73]<br>•Engman *et al*., 1989[74]<br>The studies summarize molecular characterization of the recombinant flagellar antigen, from epimastigotes, cloned and expressed in *E coli.* F29 is a flagellar calcium-binding protein encoded by several very similar genes, highly conserved among different *T. cruzi* isolates; contributes to the rapid motility of the trypanosomes, playing a role either in flagellar structure or in $Ca^{2+}$ metabolism.<br>No details of the ELISA method or results included. |
| Xenodiagnosis | • Using two boxes with 10 *Triatoma infestans* third or fourth nymphs stage.<br>• Application of boxes for 30 minutes to the skin of patient.<br>• After 30 and 60 days, examination of intestinal contents of nymphs by compression, dissection, or liquefied was performed. | Cerisola *et al*., 1974[70]<br>English translation of the paper not available. |

*Performance of a serological test was compared with another serological test; none of them used parasitological examination for comparison.

** (b) (4) (b) (4) The information in the Laboratory standardization manual[68] is based on the Laboratory manual dated 1999.

[!]The clinical features as well as the diagnosis of both leishmaniasis and African Trypanosomiasis are distinct from Chagas disease.

FITC-fluorescein isothiocyanate; OD=optical density

Table B: Study 2 (de Andrade *et al*., 1996[2]) – Summary of methods in the Lab standardization report and differences with the information available from cross-referenced publication.

| Test | Based on Lab standardization report | Differences in the method from that in the cross-referenced publications |
|---|---|---|
| IHA* | • Same as that for Study 1 except that aldehyde-preserved human RBCs, sensitized with *T. cruzi* cytoplasmic and membrane antigens were used.<br>• Observation - RBCs are agglutinating and readout.<br>• Cut-off: titer ≥ 1:16 considered seropositive. | Camargo *et al*., 1973[75]<br>Publication not available.<br><br>de Andrade *et al*., 1992[76]<br>• Cut-off: titer ≥1:10 considered seropositive. |
| IFA* | • Preparation of microscopic slides with *T. cruzi* antigen (formalized culture forms).<br>• Incubated slides with serum and controls.<br>• Detection with anti-human-IgG labelled with FITC, diluted in Evans Blue.<br>• Readout fluorescence in microscope.<br>• Cut-off: titer ≥1:40 considered seropositive. | de Andrade *et al*., 1992[76]<br>• Cut-off: titer ≥ 1:20 considered seropositive. |
| **ELISA** | | |
| Conventional*<br><br>Whole cultured parasite extract (probably epimastigotes) | • Same as for Study 1 except OD measured at 400 nm.<br>• Cut-off: OD >0.4 considered seropositive; children had to show an ELISA index of 1.2 to be considered seropositive. | Voller *et al*., 1975[72]<br>• Peru strain of *T. cruzi.* Stage not specified.<br>• Rabbit-anti-human immunoglobulin labelled with alkaline phosphatase and alkaline phosphatase substrate used. OD at 400 nm.<br>• All the 26 patients with positive xenodiagnosis were positive by ELISA.<br><br>de Andrade *et al*., 1992[76]<br>• Cut-off: Index 1.2 considered seropositive. |
| AT-CL** (Non-conventional)<br><br>AT is a glycoprotein from the trypomastigote stage | • Mucin-like trypomastigote glycoconjugates coated to an ELISA plate.<br>• Incubated with human sera at dilution 1:2000.<br>• Washing step to remove unbound material.<br>• Incubated with anti-human IgG conjugated with peroxidase for detection.<br>• Following a washing step enzyme reaction was developed with luminol (ECL reagent) and chemiluminescence read-out in luminometer.<br>• The cut-off was defined as "ten times negative control mean minus background control mean for each plate and positive serum samples gave ratios greater than 1.0.<br>• This test is based on the reactivity of serum with a purified mucin-like glycoconjugate anchored by glycosyl-phosphatidylinositol from cell-cultured trypomastigotes of the Y strain (antigen trypomastigote [AT] ELISA). The method described previously16 was modified to use direct luminometer readings of the ELISA plates. | Almeida *et al*., 1994[77]<br>• Characterization of F2/F3 component of the trypomastigotes extracted and purified from the Y and G strains of *T. cruzi.*<br>• Details of method not included.<br><br>Almeida *et al*., 1997[78]<br>• Antigens used-Trypomastigotes of the Tulahuen and Y strains.<br>• Sensitivity and specificity 100%; all of the 100 cases of confirmed *T. cruzi* infection, which had been diagnosed by one or more positive hemocultures and/or xenodiagnosis, were also positive in by CL-ELISA using the purified AT antigen, even with highly diluted sera (1:2000). Unlike the conventional ELISA using epimastigote antigen, no cross-reactivity with heterologous sera, from patients with other pathologic conditions or who had been submitted to unrelated immunization procedures was observed. It is well known that crude epimastigote antigenic preparations such as EpEx are recognized by serum antibodies present in patients with leishmaniasis.[!] |

* WHO Reference Laboratory for Chagas' disease serology at the Federal University of Goiás, Brazil

**Non-conventional AT-CL-ELISA performed at the Federal University of São Paulo R Sena Madureira, 1500 – Vila Clementino, São Paulo - SP, 04021-001, Brazil. This is the same laboratory as that of the cross-referenced publication (Almeida *et al*., 1997[78]).

[!]The clinical features as well as the diagnosis of both leishmaniasis and African Trypanosomiasis are distinct from Chagas disease.

FITC= fluorescein isothiocyanate; AT-CL-ELISA=antigen trypomastigotes chemiluminescence ELISA

Table C: Study 3 (Molina *et al*., 2014[3]) – Summary of methods in the Lab standardization report and differences with the information available from cross-referenced publication.

| Test | Based on Lab standardization report | Differences in the method from that in the cross-referenced publications |
|---|---|---|
| ELISA* (Conventional)<br><br>ORTHO® *T. cruzi* ELISA Test System (Ortho-Clinical Diagnostics, USA).<br><br>*T. cruzi* lysate | • Antigen coated to microtiter plates and incubated with diluted test samples and controls; plates washed.<br>• Incubated with a murine monoclonal antibody labelled with peroxidase and plates washed. After enzyme substrate reaction, readout in a spectrophotometer. OD measured at a wavelength of 490 or 492 nm.<br>• The cut-off is defined here as mean OD of the positive calibrator multiplied by 0.425.<br>• Specificity ranged between 99.0- 99.99% based on evaluation in different studies. The sensitivity reached values between 98.9 and 100%. | Commercially available assay used |
| ELISA* (Non-conventional)<br><br>Bioelisa Chagas (Biokit, Spain)<br><br>Recombinant antigen consisting of four immunodominant epitopes of *T. cruzi*, which is licensed by (b) (4) | • The plasma or serum samples directly added to the wells and incubated; followed washing steps to remove the unbound antibodies and a rabbit anti-human-IgG/IgM labelled with horseradish peroxidase for detection of formed antigen-antibody complexes. Plates washed and the enzyme substrate solution added and reaction stopped with sulfuric acid. The read-out performed by a spectrophotometer at 450 nm.<br>• The cut-off value is defined as mean absorbance of the negative controls plus 0.300 and the sample is positive if the absorbance divided by the cut-off gives a ratio over 1.0.<br>• Sensitivity 100%; the specificity values range between 97.4 to 99.5%. | Commercially available assay used |
| RT-PCR*<br><br>Testing performed in the same laboratory as the cross-referenced publication | • Blood samples treated with guanidine hydrochloride 6M and DNA extracted.<br>• 3 primers used to amplify a 166 bp segment in the satellite DNA of *T. cruzi*:<br>o Cruzi 1 (forward-nucleotide position 27-46): ASTCGGCTGATCGTTTTCGA<br>o Cruzi 2 (reverse-nucleotide position 172-192): AATTCCTCCAAGCAGCGGATA.<br>o Cruzi 3 (The TaqMan probe-nucleotide position 143-159; labeled with 5´FAM (6-carboxy-fluorescein) and 3´MGB (minor groove binder): CACACACTGGACACCAA<br>• Amplification performed in duplicate in a thermocycler SmartCycler (Cepheid, USA)<br>• Internal (TaqMan Human RNase P detection reagent; Applied Biosystems) and external (known positive or negative samples).<br>• If both threshold (CT) were <40, the sample was considered positive. With a CT value >45 the sample was considered negative (Hospital Vall d´Hebron, *T. cruzi* RT-PCR procedure).<br>• The lab used for this PCR testing took part in an external quality assurance (EQA) program to assess the agreement of qPCR performances between laboratories (Ramirez et *al*., Manuscript included). | Molina *et al*., 2014[3]:<br>•DNA was extracted from 200 µL of blood.<br>•Amplification was performed in duplicate, according to a method (Piron *et al*., 2007[82]). A sample was considered to be positive when the cycle threshold of both amplifications was less than 40 and negative when the cycle threshold of both amplifications was higher than 45. If the results of amplifications were discordant or cycle thresholds were between 40 and 45, amplifications were repeated and were considered to be positive when at least one cycle threshold result was less than 40.<br><br>Piron *et al*., 2007[82]<br>Specificity: 100%<br>Sensitivity: 0.8 parasites/mL (50% positive hit rate) and 2 parasites/mL (95% positive hit rate); this is based on serial dilution of a samples spiked with epimastigotes. A negative result in the RT-PCR assay should be reported inferior to 2.8 parasites/mL. |

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[1]Paralell PCR tests (control) performed in (b) (4) (b) (4).

Table D: Study 4 (DNDi-CH-E1224-001) – Summary of methods in the Laboratory standardization manual[68], test package inserts and differences with the information available from cross-referenced publication if applicable.

| Test | Based on Lab standardization report and the test package insert |
|---|---|
| **IHA**<br>(b) (4)<br>*T. cruzi* cytoplasmic and membrane antigens<br>Two study sites[1, 2] Baseline (for screening only) | • A stabilized suspension of ram erythrocytes (sheep or chicken) used with the characteristic of being able to agglutinate sensitized red blood cells.<br>• Serial dilutions of patient's serum are added to a microtiter plate with the red blood cells carrying *T. cruzi* antigens.<br>• The titer was defined as the highest serum dilution presenting agglutination and should be compared to the titer of positive and negative control.<br>• 98% sensitivity if a titer ≥16 is defined as reactive, 100% sensitivity if a titer of ≥8 is defined reactive (according to package leaflet. 100% specificity if titer of ≥16 is defined as reactive. |
| **IFA**<br>Baseline (for screening only) | • No details included in the Lab standardization report |
| **ELISA (Conventional)**<br>Chagatest Lysate ELISA from (b) (4) (b) (4)<br>Cytoplasmic and membrane antigens of *T. cruzi.*<br>Study sites[1, 2]: Baseline (for screening only) | • Antigen coated to microtiter plates and incubated with diluted test samples and controls; plates washed. Incubated with a goat-antihuman IgG antibody labelled with peroxidase and plates washed. After enzyme substrate reaction, readout in a spectrophotometer. OD measured at a wavelength of 450 nm.<br>• Run validation: run was valid if the following two conditions were met simultaneously:<br>o The readings of at least 2 out of the 3 negative controls (NC) should be ≤ 0.150 OD<br>o The mean reading of the positive controls should be 0.600 OD.<br>• The cut-off defined as the average of the negative control OD readings plus 0.200 .<br>• Grey zone: cut-off ± 10%<br>• Senstivity-100% assuming a prevalence of 100% in the presence of specific IgG antibodies detectable by other techniques (IIF, HAI, DA).<br>• Specificity-99.2 -99.6%; specificity >99%. |
| **ELISA (Conventional)**<br>Chagatek ELISA from (b) (4) (b) (4)) (Chagatek ELISA. (b) (4), (b) (4)<br>Study site[1, 2]: Baseline (for screening only)<br>Study site[3]: Follow-up | • Method and principle of the assay same as above.<br>• The cut-off defined here as the mean of the negative control readings plus 0.100 OD.<br>• Sensitivity and specificity -100% assuming a prevalence of 100% in the presence of specific IgG antibodies detectable by other techniques (IFA, IHA, DA). |
| **Recombinant ELISA (Non-conventional)**<br>(b) (4) (Chagatest ELISA recombinante v.3.0. (b) (4)<br>Recombinant *T. cruzi* antigen (obtained using recombinant DNA techniques from proteins from amastigote and trypomastigote forms of *T. cruzi)*<br>Study sites[1, 2]: Baseline (for screening only)<br>Study site[3]: Follow-up | • The cut-off defined as average negative control readings plus 0.300 OD.<br>• Sensitivity-100% in samples with positive xenodiagnosis and serology, 99.3% sensitivity in samples with positive serology (HAI, IIF, another ELISA) (according to package leaflet). Specificity-97.8% in samples with negative xenodiagnosis and negative serology, 100% specificity in samples negative by serologic testing (IHA, IFA, another ELISA). |
| **AT-CL- ELISA (Non-conventional)**<br>Detects lytic anti-α-Gal antibodies (IgG) against *T. cruzi gl*ycosylphosphatidylinositol-anchored mucins (tGPImucins).<br>Study site[4] | **From Laboratory standardization manual[68]:**<br>• Method and principle of the assay similar to above.<br>• Antibodies detected with a biotinylated sheep-antihuman IgG, washed and the color developed with streptavidin-horseradish peroxidase (the streptavidin binds to the biotinylated antibodies). After washing, a luminol containing substrate for the detection of formed antigen/antibody complexes added and the luminescence reaction measured by a luminometer.<br>• Cut-off values defined as the upper prediction limit, which is expressed as the standard deviation multiplied by a factor based on the number of negative controls and the confidence level (99.5%) (Izquierdo *et al*., 2013[83]).<br>**From Cross-references**<br>(Almeida *et al*., 1997[78]; Izquierdo *et al*., 2013[83]; de Andrade *et al*., 1996[2])-For details see Table B above.<br>Sensitivity-100% and Specificity-100% in the diagnosis of confirmed Chagas disease; no cross reactions with patients with leishmaniasis or other infectious or autoimmune disease. |

[1] Plataforma de Atención Integral a los Pacientes con Enfermidad de Chagas, Av. Aniceto Arce casi Av. Oquendo, Cochabamba, Bolivia
[2] Plataforma de Atención Integral a los Pacientes con Enfermidad de Chagas, Calle España esquina Pasaje California, Tarija, Bolivia
[3] Laboratorio de la PLataforma de Atención al Paciente con Chagas-Cochabamba, Av. Aniceto Arce N 371, casi Pasteur, Cochabamba, Bolivia.
[4] CRESIB (Spain) - Dr. Izquierdo Instituto de Saludad Global de Barcelona, c/ Rosselló, 132, 5 2ª (08036) Barcelona.
(CRESIB - Centre de Recerca en Salut Internacional de Barcelona International Health Center, Hospital Clinic de Barcelona, Barcelona, Spain.)

Table E: Study 4 (DNDi-CH-E1224-001) – Summary of RT-PCR method* based on the Lab standardization reports and cross-referenced publication.

The method for the multiplex RT-PCR (MTq-qPCR) similar to that for Study 3 based on the amplification of the satellite DNA of *T. cruzi* (for details see Table C). Some of the modifications are as follows:

- Amplification of an internal amplification control (IAC) using the following primers and probes with the same melting temperature:
  - **IAC Tq Fw**
    5'-ACCGTCATGGAACAGCACGTA- 3'
  - **IAC Tq Rv**
    5'-CTCCCGCAACAAACCCTATAAAT- 3'
  - **IAC (Probe)**
    5'-AGCATCTGTTCTTGAAGGT- 3'
- The DNA extraction performed with the High Pure PCR Template Preparation Kit (Roche Applied Science, USA) and a linearized pZErO plasmid containing a sequence of Arabidopsis thaliana was added for an internal amplification control (IAC).
- The patient was RT-PCR positive when the CT value was <40. The limit of quantification was 1.5 parasite equivalents/mL and values higher than the limit of quantification were quantified from the calibration curve.
- To analyze the performances of existing qPCR methods recently an external quality assurance (EQA) program was established with the help of three labs (Ramirez et al., Manuscript in preparation). One of the labs taking part was the one performing the qPCR tests for this trial as well as the lab used for PCR testing in Study 5 (CHAGASAZOL01).
- The agreement of the qualitative results was evaluated using "four proficiency testing panels containing seronegative blood samples spiked with 1, 10 and 100 parasite equivalents/mL of four Trypanosoma cruzi stocks, and four negative controls were prepared by the (b) (4)
  Furthermore, blood samples of patients from the DND-CH-E1224-001 trial were sent to (b) (4) and tested again to evaluate the rate of agreement between laboratories. This EQA showed that the congruence of qualitative results between and within the three laboratories was very high. The results of the retested blood samples showed no significant differences concerning quality or quantity of DNA as well. This study also demonstrated that blood samples treated with guanidine-EDTA buffer for 48 hours and conserved at 4 °C for 12 months were stable. Thus, it is justified to use qPCR testing as a therapeutic response endpoint (Ramirez et *al*., Manuscript in preparation).
- Sensitivity-83.3-94.4%; specificity 85-95%.
- LOD 10 fg/µL.
- Limit of quantification: 1.5 par eq/mL (SOP study manual).
- The sample is valid if:
  - The internal amplification control (yellow channel) is amplified with an efficient signal.
  - The sample is positive or *T. cruzi* is detected if the fluorescent curve in the green channel crosses the threshold leading to a Ct value.
  - The sample is negative or *T. cruzi* is not detected if: The fluorescent curve does not cross the threshold leading to the absence of a Ct value.
- Detectable *T. cruzi* positive sample and control: when the fluorescence curve intersects the threshold with Ct < 40.
- Undetectable *T. cruzi* negative sample and control: when the fluorescence curve does not intersect the threshold resulting in no Ct or when it intersects the threshold with Ct > 40.
- Reporting of sample results
  - Ct values > 40: undetectable.
  - Values below the limit of quantitation (1.5 parasite equivalents/mL): "detectable but not quantifiable".
  - Values higher than or equal to the limit of quantitation: detectable and quantifiable from the calibration curve.
- For visits at which 3 blood samples (3 × 10 ml) are taken, the result were considered positive when at least one of the 3 qPCR reactions has a positive result (i.e. detectable; quantifiable or not quantifiable). The result were considered negative when the qPCR result of the 3 samples is negative (i.e., undetectable).

*Testing performed in the (b) (4)
(b) (4).
External QC check was performed in (b) (4)
(b) (4)

Fw-forward; Rv-reverse

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06/12/2017

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