Anti-parasitic effect of vitamin B\textsubscript{12} on \textit{Trypanosoma cruzi}.

Running title: Anti-\textit{T. cruzi} activity of vitamin B\textsubscript{12}.

ALEJANDRA B. CICCARELLI,\textsuperscript{a,}\textsuperscript{†} FERNANDA M. FRANK,\textsuperscript{b,}\textsuperscript{c,}\textsuperscript{†} VANESA PUENTE,\textsuperscript{a} EMILIO L. MALCHIODI,\textsuperscript{b,}\textsuperscript{c} ALCIRA BATLLE,\textsuperscript{a,*} and MARIA ELISA LOMBARDO\textsuperscript{a,}\textsuperscript{d,*}.

\textsuperscript{a}Centro de Investigaciones sobre Porfirinas y Porfiriás, CIPYP (UBA-CONICET), Hospital de Clínicas José de San Martin, UBA, Buenos Aires, Argentina. \textsuperscript{b}Departamento de Microbiología, Facultad de Medicina, UBA, Buenos Aires, Argentina. \textsuperscript{c}Cátedra de Inmunología, IDEHU (UBA-CONICET), Facultad de Farmacia y Bioquímica, UBA, Buenos Aires, Argentina. \textsuperscript{d}Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, UBA, Buenos Aires, Argentina.

*Corresponding authors:

Lombardo María Elisa. Int. Guiraldes 2160, Facultad de Ciencias Exactas y Naturales, 2\textsuperscript{a} Piso CM1, CABA, Buenos Aires, Argentina (CP-1428).

Telephone: 54-11-4812-3357. Fax: 54-11-4811-7447

E-mail: elombardo@qb.fcen.uba.ar

Alcira Batlle. Viamonte 1881, 10 “A”, Buenos Aires, Argentina (CP-1056)

Telephone: 54-11-4812-3357. Fax: 54-11-4811-7447.

E-mail: batllealcira@yahoo.com.ar

† A. C. and F.M.F. contributed equally to this work.
Abstracts

A nutritional characteristic of trypanosomatid protozoa is that they need a heme-compound as a growth factor. Because of the cytotoxic activity of heme and its structural similarity with cobalmins, we have investigated the \textit{in vitro} and \textit{in vivo} effect of vitamin B\textsubscript{12} (B\textsubscript{12}, cyanocobalamin) on the different forms of \textit{Trypanosoma cruzi}. Cyanocobalamin showed a marked anti-parasitic activity against epimastigote (IC\textsubscript{50} 2.42 µM), amastigote (IC\textsubscript{50} 10.69 µM) and trypomastigote (IC\textsubscript{50} 9.46 µM). Anti-epimastigote and -trypomastigote values were 1.7 to 4 times lower than those obtained with benznidazole (reference drug). We also found that B\textsubscript{12} and hemin do not interact with each other in mode of action. Our results show that B\textsubscript{12} increases intracellular oxidative activity and stimulates both superoxide dismutase (50%) and ascorbate peroxidase (20%) activities whilst activity of trypanothione reductase was not modified. In addition, we found that the antioxidants dithiothreitol or ascorbic acid increase the susceptibility of the parasite to the cytotoxic action of B\textsubscript{12}. We propose that vitamin B\textsubscript{12} would be exerting its growth inhibitory effect through the generation of reactive oxygen species. In an \textit{in vivo} assay, a significant reduction in the number of circulating parasites was found in \textit{T. cruzi}-infected mice treated with cyanocobalamin and ascorbic acid. The reduction of parasitemia in benznidazole treated mice was improved by the addition of these vitamins. According to our results, a combination of B\textsubscript{12} and Bnz may be further investigated due to its potential as a new therapeutic modality for the treatment of Chagas' disease.

Keywords: \textit{Trypanosoma cruzi}; Antiparasitic agents; Vitamin B\textsubscript{12}; Antioxidant enzymes.
Abbreviations: APx: ascorbate peroxidase; B12: Vitamin B12; Bnz: Benznidazole; CD: cellular density; DCF: diclorofluorescein; DMSO: dimethyl sulfoxide; CPRG: Chlorophenol Red-β-D-galactopyranoside; DTT: dithiothreitol; H2DCFDA: 2',7'-dichlorodihydrofluorescein diacetate; PBS: phosphate buffered saline; ROS: reactive oxygen species; SOD: superoxide dismutase; Try: trypanothione; TryR: trypanothione reductase; WHO: World Health Organization.
Introduction

Trypanosoma cruzi is the causative agent of Chagas’ disease, which is a major endemic disease in Latin America. Current treatments employ benznidazole (Bnz) or nifurtimox, two drugs that have been in use for over 40 years. The chemotherapy for this disease is not satisfactory due to the limited efficacy and the toxicity associated with long-term treatments (16, 17). Research and development of new drugs effective in the treatment of this disease, that affect 16–18 million people in the Americas (25), is a real need and requires new strategies for drug development (2, 22).

Heme-compounds are necessary as growth factor for T. cruzi (7). However, hemin and related porphyrins have an important cytotoxic action through the generation of reactive oxygen species (ROS) such as superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and the highly reactive hydroxyl radical (OH·, 6). We have previously studied the effect of hemin on growth and the antioxidant defense system in T. cruzi epimastigote (4) demonstrating the correlation between higher hemin concentrations in the culture medium and oxidative damage in the cells. Above 15 µg/ml hemin produced a clear decrease in growth rate, inducing the transformation of epimastigotes into amastigotes accompanied by a marked injury on the antioxidant enzymatic machinery of the parasite (4). Similar results have been reported in Leishmania donovani promastigotes cultured in vitro (15).

Since the structures and uptake systems of heme and cobalamin are alike (5), and the known cytotoxic and antitumor activity of cobalamins administered either alone or combined with antioxidants are also similar (11, 18, 19, 23), it was of great interest to investigate the effect of cyanocobalamin (vitamin B$_{12}$) on T. cruzi.
Here, we have evaluated the in vitro antiparasitic activity of vitamin B\textsubscript{12} against different forms of the \textit{T. cruzi}, and we have explored its possible mode of action. Additionally we have analyzed the interaction of B\textsubscript{12} with hemin and the improvement in the activity by the addition of antioxidants. More importantly, we have investigated the capacity of B\textsubscript{12} to control parasitemia, in the murine model, showing its effect combined with the Bnz treatment as a novel therapeutic modality.

**Materials and methods**

**Chemicals**

Hemin, NADPH, EDTA, NADH, Chlorphenol Red-β-D-galactopyranoside (CPRG), RPMI-1640 Medium, hydrogen peroxide, ascorbic acid and 2', 7'-dichlorodihydrofluorescein diacetate (H\textsubscript{2}DCFDA) were obtained from Sigma Chem. Co. (Saint Louis, MO, USA). Yeast extract, tryptose, powered beef liver and brain heart infusion were from Difco Laboratories (Sparks, MD, USA). Trypanothione was purchased from Bachem Bioscience Inc. (USA). Benznidazole (Bnz) was kindly provided by Roche (Argentina). All other chemicals were of the highest purity commercially available.

**Parasites**

\textit{Trypanosoma cruzi} epimastigotes (Tulahuen strain) were grown at 28 °C in a liquid medium containing 0.3% yeast extract, 0.9% tryptose, 0.4% dextrose, 1% disodium phosphate 2-hydrate, 0.36% sodium chloride, 0.04% potassium chloride, 0.15% powered beef liver, 0.5% brain heart infusion and 0.5-1.0 mg/100 ml hemin. \textit{T. cruzi} bloodstream trypomastigotes were obtained from infected CF1 mice by cardiac puncture, at the peak of parasitemia on day 15.
postinfection. Trypomastigotes were routinely maintained by infecting 21 days-old CF1 mice. 

*T. cruzi* parasites from the Tulahuen strain stably expressing the β-gal gene were kindly provide by Dr. Buckner (3).

**Animals**

Outbred CF1 male and inbred C3H/HeN female mice were nursed at the Departamento de Microbiología, Facultad de Medicina, Universidad de Buenos Aires. Animals were treated in accordance with guidelines established by the Animal Care and Use Committee of the Argentine Association of Specialists in Laboratory Animals (AADEALC).

**In vitro assays for anti-*T. cruzi* activity**

To evaluate the growth inhibition of *T. cruzi* epimastigotes, parasites from a 3 days-old culture were inoculated into fresh culture medium to reach an initial concentration of 1.5 - 2.5 x 10^7 cells/ml. Cells were cultured in the presence of 0.125 to 15 µM of B12 or 0.75 to 25 µM of Bnz (used as positive control) for 3 days (or the time indicated in the experiment). The compounds ability to inhibit growth of the parasite was evaluated, in triplicate, in comparison to the control without drug. Cells growth was followed by counting the number of cells per ml of culture using a Neubauer chamber and was expressed as cellular density (CD). The percentage of inhibition (%I) was calculated as: %I = \{1 - ((CD_{3t} - CD_0) / (CD_{3c} - CD_0))\} x 100, where CD_{3t} is cellular density of treated parasites at day 3; CD_0 is cellular density of parasites at day 0 and CD_{3c} is cellular density of untreated parasites (control) at day 3. To evaluate the combinatory effect of B12 and Bnz, the IC_{50} values for Bnz in the presence of different B12 concentrations (0.8 to 2.4 µM) were calculated. The inhibitory activity of B_{12} (0.25 µM) was also evaluated.
in the presence of antioxidant agents such as dithiothreitol (DTT; 0.5-1 µM) and vitamin C (C) or ascorbic acid (0.5-1.5 µM).

The trypanocidal effects were also tested on bloodstream trypomastigotes according to a standard WHO protocol with minor modifications (20, 21). Briefly, parasites were counted in a Neubauer chamber and the blood was diluted in RPMI culture medium to a final concentration of 1.5 × 10⁶ trypomastigotes/ml. Parasites were seeded (150 µl/well) in duplicate in a 96-well microplate, in the presence of B₁₂ (0.37 to 72 µM) or Bnz (0.38 to 38 µM). Plates were incubated for 24 h, and the remaining alive parasites were counted in a Neubauer chamber. The results were expressed as the percentage of lysed parasites (%L) relative to the number of parasites in the control (without adding the drug).

Amastigote growth inhibition assay was performed on ninety-six well tissue culture plates seeded with a murine macrophage cell line, J774, at 5 × 10³ per well in 100 µl complete RPMI medium without phenol red and incubated 2 h at 37°C 5% CO₂. Cells were infected with transfected trypomastigotes expressing β-galactosidase, at a parasite cell ratio 10:1 as previously describe (13). After 24 h of co-culture, plates were washed to remove unbound parasites and drug compounds were added in duplicate. Controls included uninfected J774 cells (0% infection control) and cell monolayers infected with trypomastigotes (100% infection control). On day 7, the assays were developed by addition of CPRG (100 µM final concentration) and Nonidet P-40 (1% final concentration). Plates were incubated for 4 to 6 h at 37°C. Wells with galactosidase activity turned substrate from yellow to red, and this was quantified using a microplate reader (A₅₇₀ nm, Bio-Rad Laboratories). The percentage of inhibition (%I) was calculated as 100 – \{[(Absorbance of treated infected cells - Absorbance of treated uninfected cells)/(Absorbance of untreated infected cells)] x 100\}, and the IC₅₀ was estimated. Because the B₁₂ is coloured and show a significant absorbance
at 570 nm, blanks of uninfected cells with the different doses of B12 were done and their absorbances were subtracted.

Cytotoxicity assay

Cytotoxic activity was evaluated in vitro, using Vero cells cultured under the standard conditions. Cells, 9 x 10^5 cell/ml were seeded in a 24-well plate and after 48 h different concentrations of B12 (6 - 2400 µM) or Bnz (3 - 3000 µM) were added. After 24 h of incubation, cells were washed twice with PBS and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added at a final concentration of 0.5 mg/ml. Plates were incubated for 1h at 37°C. Finally, blue precipitates were dissolved in 0.5 ml of dimethyl sulfoxide (DMSO) and were read on a plate reader (Spectra Count™ BS 10001) at a wavelength of 570 nm. Values from blank wells containing only medium and reagents were subtracted from the values of the samples. The values of absorbance showed a good correlation with viable cells counts using trypan blue. All MTT assays were repeated at least three times by using four samples per assay. The selectivity index (SI) was calculated as the 50% cytotoxic concentration on Vero cells divided by the IC_{50} of the compound for T. cruzi cells.

Assay of intracellular oxidative activity

The intracellular oxidative activity was assessed using the oxidant-sensitive fluorescent probe H2DCFDA. After 3, 7 or 24 h of treatment with 15, 30 and 60 µM of B12 (these conditions are referred from now on, as short-time treatment), the parasites were harvested, suspended in PBS at a concentration of 1 x 10^6 cells/ml and stained for 30 min in the dark at 37 °C with 10 µM H2DCFDA taken from a 0.2 mM stock solution in DMSO. Then, the fluorescence of
dichlorofluorescein (DCF) in cells was analyzed by Becton Dickinson FACScalibur flow cytometer with an excitation wavelength of 480 nm and an emission wavelength of 530 nm. The flow cytometry results were expressed by the ratio Gm_t/Gm_c, where Gm_t and Gm_c correspond to the geometric mean of histograms obtained for treated and untreated (control) cells respectively.

Enzymatic determinations

All steps were performed at 2-4°C. Parasites (200 ml of culture) coming from short-time treatment were harvested by centrifugation at 12000 g for 10 min, washed once and resuspended in 5 ml of: a) Tris-HCl buffer for APx activity, b) sodium phosphate buffer for SOD activity and c) potassium phosphate buffer for TryR activity. Cells in suspension were disrupted by sonication in a Soniprep 150, MSE Ultrasonic Power for 45 sec. The resulting homogenate was centrifuged at 5000 g for 15 min, the precipitate discarded and the supernatant employed as source of enzymes. Protein concentration was determined according to the method described by Lowry (8); and these values were considered to express the enzymatic activities as specific activities (EU/mg of protein).

Superoxide dismutase (SOD) activity has been assayed by a spectrophotometric method based on the inhibition of a superoxide-driven NADH oxidation as previously described (4). One enzyme unit (EU) is defined as the amount of protein required to inhibit 50 % NADH oxidation.

Ascorbate peroxidase (APx) activity was measured following the change in absorbance at 265 nm due to ascorbate oxidation, at 25 ºC (4). Enzyme activity was calculated by using an ε value of 16.00 x 10^3 M^-1 cm^-1. The EU is defined as the amount of enzyme forming 1 nmol of
product per sec under the standard incubation conditions.

Trypanothione reductase (TryR) activity was determined following NADPH oxidation at 340 nm, at 25 ºC (1). The activity was calculated using an extinction coefficient of $6.22 \times 10^3$ M$^{-1}$ cm$^{-1}$. One EU is defined as the amount of enzyme forming 1 nmol of product per minute under the standard incubation conditions.

**In vivo trypanocidal activity assay**

Groups of five C3H/HeN mice (6 to 8 weeks old) maintained under standard conditions were infected with $5 \times 10^3$ bloodstream *T. cruzi* trypomastigotes by the intraperitoneal route. Five days after infection, the presence of circulating parasites was confirmed by microhematocrit method. Mice were treated with B$_{12}$ (1.5 mg/kg of body weight/day), B$_{12}$ (1.5 mg/kg of body weight/day) plus ascorbic acid (1.5 mg/kg of body weight/day), Bnz (0.75 mg/kg of body weight/day) or the combination of the treatments. The administration of the drugs was performed from Monday through Friday during two weeks (days 5 to 9 and 12 to 16 postinfection), by the intraperitoneal route. Drugs were resuspended in 0.1 M phosphate-buffered saline (PBS, pH 7.2), and this vehicle was employed also as a negative control. In addition, control groups receiving ascorbic acid (1.5 mg/kg of body weight/day) or the combination of both but half doses (0.75 mg/kg of body weight/day each) were included. Levels of parasitemia were monitored every 2 days in 5 µl of blood diluted 1:5 in lysis buffer (0.75% NH$_4$Cl, 0.2% Tris, pH 7.2) by counting parasites in a Neubauer chamber. The number of deaths was recorded daily.

**Statistical analysis**
All data are expressed as means ± SEMs, parasitemia is also expressed as Area Under the Curve. To calculate the IC$_{50}$ values, the %I or %L values were plotted against the log of drug concentration (µM) and fitted with a straight line determined by a linear regression (Sigma Plot 10 software). The fractional inhibitory concentrations (FICs) were calculated as the ratio of the IC$_{50}$ of the drug in combination and the IC$_{50}$ of the drug alone. The FIC index (FICI) for two drugs was the FIC of drug B$_{12}$ plus the FIC of drug Bnz. The significance of differences was evaluated using Student’s $t$ test, taking $p < 0.05$ as significant. The results presented are representative of three to four independent experiments. Survival curves were compared with Log-rank Test.

**Results**

*In vitro antiparasitic activity*

*T. cruzi* epimastigotes were grown during 7 days in culture medium containing 10 µg/ml hemin plus different concentrations of B$_{12}$ (Fig. 1) and cells were counted daily. All the tested B$_{12}$ concentrations produced a decrease in the growth rate comparing to control in a dose-dependent manner, as observed on the 3$^{rd}$ day. Concentration of B$_{12}$ as high as 45 µM presented trypanocidal activity showing a negative slope until day 3. For longer times, growth rates were similar showing that the B$_{12}$ antiparasitic effect only last for short periods of time, which may be explained by instability of B$_{12}$ in the culture.

Independently of the doses and exposure time, B$_{12}$ did not produce morphologic changes, as observed by optical microscopy, but motility was markedly diminished (data not shown). The inhibitory effect of B$_{12}$ was manifested independently of the hemin concentration added to the cultures (Fig. 2). To corroborate if the parasites were still B$_{12}$ susceptible after 4 days of...
treatment, a new addition was made showing a dose dependent response. The results obtained in absence of B12 corroborate the inhibitory effect of high concentrations of hemin (30 µg/ml) on the growth of *T. cruzi* epimastigotes already reported (4) (Fig. 2).

We next analyzed the inhibitory activity of B12 and Bnz, the drug currently used for treatment of Chagas disease, against the three stages of *T. cruzi* (Table 1). Similar IC$_{50}$ values for B12 on trypo- and amastigotes forms were found. B12 was found to be 1.7 to 3.6 and 2.6 to 4 times more active than Bnz on the epimastigote and trypomastigotes forms respectively.

In epimastigotes the combined effect of B12 and Bnz was also investigated. The isobologram depicted in Fig 3 shows an additive effect for the combination of both drugs.

Cytotoxicity assay

The results of the cytotoxic activity of the B12 or Bnz on Vero cells are shown in Fig. 4. For B12 concentrations as high as 2400 µM no cytotoxic effect was found, while for Bnz the 50% cytotoxic concentration was 82.79 ± 2.75 µM. The SI was employed to compare the toxicity for mammalian cells and the activity against the parasites. The SIs for the epimastigote form of *T. cruzi* were > 991.7 for B12 and 14.1 for Bnz, while for the trypomastigote forms, values of > 253.7 for B12 and 2.7 for Bnz were found. For the amastigote form, the SI value for B12 was 224.5.

Short-time treatment and action mode

To elucidate the mechanism of action, high concentrations of B12 (15 to 60 µM) and short-time treatment (3 to 24 h) on epimastigotes of *T. cruzi* were employed. We expected that B12 by analogy with hemin, would act by inducing the generation of ROS. Results obtained
by flow cytometry to evaluate intracellular oxidative stress are shown in Fig. 5. Independently of B₁₂ concentration the fluorescence of H₂DCFDA-loaded epimastigotes increased ~12 to 14 times after only 3 h of treatment and was kept markedly high (~16-18 times) until the end of treatment (24 h). The addition of 0.2 mM H₂O₂ as a positive control caused a similar increase of around 15 times in cell fluorescence intensity (data not shown). Because of evidence for oxidative stress on treated parasites, we studied the antioxidant enzymes activities in short-time treatments with 30 µM B₁₂ (Fig. 6). A similar behavior for SOD and APx activities was observed. During the first 3 h treatment, the oxidative damage of the enzymes is evident, presenting an equally reduced activity (around 25% below control). The antioxidant activity of these enzymes was manifested after 7 and 24 h with an increased activity for SOD (50-55% above control) and APx (20–25% above control). During the evaluated period of time, the activity of TryR was not significantly different from the control. Taken these results together, it can be considered that treatment with B₁₂ induces the generation of superoxide anion and hydrogen peroxide and that despite the increased activity of SOD and APx, the intracellular oxidative stage persists due to an incomplete metabolism of those reactive species and/or the lack of an increase in TryR activity.

Effect of antioxidant agents

To obtain further information regarding the mode of action of B₁₂, the effect of antioxidant agents such as DTT and ascorbic acid (vitamin C) was evaluated (Table 2). Employing a low concentration of B₁₂ (0.25 µM), both antioxidants enhanced considerably the antiparasitic activity in vitro. As expected (9, 10), the antioxidants showed a doses
dependent antiparasitic activity reaching 8 fold inhibition when DTT and ascorbic acid were used at 1.0 and 1.5 µM, respectively (Table 2).

In vivo antiparasitic activity

To determine whether treatments could be useful for in vivo therapies, an acute murine model was employed. Thus, 7 groups of mice were infected with T. cruzi trypomastigotes and injected with B₁₂ alone, B₁₂ plus ascorbic acid, Bnz alone and Bnz plus ascorbic acid and B₁₂. Individual parasitemia levels were assessed every other day. At the peak of parasitemia, around day 13, all treated mice presented an important decrease in the number of circulating parasites comparing with control group (p < 0.01; Fig. 7A). When B₁₂ was administrated alone, a reduction in parasitemia could be observed (2.23 ± 0.28 x 10⁶ vs Control: 4.18 ± 0.02 x 10⁶ parasites/ml), thus this reduction was improved by the administration of B₁₂ simultaneously with ascorbic acid (1.26 ± 0.17 x 10⁶ parasites/ml). Moreover, when infected animals were treated with half of the doses of the vitamins, we still observed a significant reduction in parasitemia (1.62 ± 0.23 x 10⁶ vs Control: 4.18 ± 0.02 x 10⁶; p<0.01; data not shown).

At the peak of parasitemia, mice treated with Bnz alone or combined with vitamins, presented similar levels of circulating parasites (1.43 ± 0.12 x 10⁶ and 1.33 ± 0.25 x 10⁶ parasites/ml respectively), than those treated with the vitamins. Important differences in the kinetics of parasitemia could be observed throughout the acute phase of infection. Thus calculating the area under the curve, decreases in the number of circulating parasites of 43.9%, 58.6% and 64.6% were observed for the mice treated only with vitamins, Bnz or the combination of both respectively (Fig 7A). The reduced number of parasites was crucial for animal survival, as shown in Fig. 7B. Control mice presented high levels of parasitemia.
leading to death between days 14 and 28 postinfection. In contrast, 83.3% of Bnz plus vitamins treated animals survived until the end of the experiment (100 dpi). Only animals receiving Bnz combined with vitamins presented significant survival rate comparing with control animals (p<0.05).

Discussion

The effect of B12 on the parasite T. cruzi was investigated for the first time. B12 produced a marked decrease in epimastigotes growth rate (Fig. 1), together with significant changes in the motility. Unlike hemin, which at 25 µg/ml produce the epimastigote transformation to amastigote (4), no morphological changes were observed for B12 concentration up to 45 µM. The inhibitory effect of B12 on epimastigotes growth was increased in a dose-dependent manner by a second addition of B12 at the fifth day. Previously, we have demonstrated that high concentration of hemin produce an antiproliferative effect on T. cruzi epimastigotes (4). Although the structural similarities between B12 and hemin, the antiproliferative effect of cyanocobalamin occurs regardless the hemin concentration present in the culture medium (Fig. 2). Even in the presence of B12, the trypanocidal effect of high concentration of hemin was manifested, demonstrating that B12 do not influence its effect. These results corroborate that B12 and hemin does not interact with each other on their mode of action. At the concentrations assayed, apparently there was not a competitive effect between both compounds.

Cyanocobalamin showed a marked in vitro anti-T. cruzi activity, with IC50 values of 2.42 ± 0.54 µM, 10.69 ± 1.50 µM and 9.46 ± 1.20 µM for epimastigote, amastigote and trypomastigote forms, respectively (Table 1). B12 showed activity levels between 1.7 to 4 times higher than Bnz in epimastigote and trypomastigote stages. Moreover, the effect
produced by a combination of B\textsubscript{12} and Bnz was the sum of the effects produced by the
components alone (Fig\textsuperscript{3}).

Due to the high trypanocidal activity and low cytotoxicity, cyanocobalamin presented high
SI values (>200) in all parasite stages. This is particularly significant since a SI > 50 is
considered adequate for trypanocidal drugs under development (14).

The short-time treatment of \textit{T. cruzi} epimastigotes with high doses of B\textsubscript{12} (15 - 60 µM)
showed a significant increase in the cellular oxidative state (Fig.\textsuperscript{5}). Superoxide anion
generation may cause the increased activity of SOD (50% above control value, Fig.\textsuperscript{6})
which would transform the superoxide anion into hydrogen peroxide. Under these
conditions, it was expected that hydrogen peroxide metabolizing enzymes would increase
their activity. Although catalase and selenocysteine-containing glutathione peroxidases are
absent in \textit{T. cruzi} (24), the parasite has an efficient, redundant, and ubiquitously distributed
antioxidant defense system. The enzymes APx and tryparedoxin peroxidase would be
responsible for degrading the hydrogen peroxide (24). In this work we have evaluated the
TryR activity, as an indirect way to measure APx and tryparedoxin peroxidase activities
finding that there were not significant differences from the untreated control (Fig.\textsuperscript{6}). TryR
is an enzyme that is likely to be pivotal to peroxide metabolism in all trypanosomatids,
because reduced Try is the molecule reducing dehydroascorbate to ascorbate, and oxidized
tryparedoxin to reduced tryparedoxin (12). The lack of increased activity of TryR above
control and the slight increase of APx activity could be responsible for accumulation of
hydrogen peroxide within the cell. This excess of hydrogen peroxide and the rest of
superoxide anion that may not have been metabolized by SOD, would be the species
responsible for intracellular oxidative stress induced by B\textsubscript{12}. A deleterious action of B\textsubscript{12}
treatment only became evident for SOD and APx (activity values significantly lower than
control), at 3 h of treatment with B_{12} (Fig. 5). At later times the antioxidant action of these enzymes was manifested but it was not enough to reconstitute the cellular equilibrium.

As was observed with hemin (4), vitamin B_{12} appears to express its cytotoxic action on the parasite through the generation of ROS. By adding antioxidants, as DTT or Vit. C, the oxidative damage raises instead of decreasing, probably due to the well-known prooxidant effect of these compounds combined with transition metals ions (Fe, Cu, Co), which makes itself evident through the generation of ROS (18, 19). Concerning to the possible mechanism of action of B_{12}, additional studies should carried out. The oxygen-reduction products have been implicated in the mechanism of action of several trypanocidal agents active \textit{in vitro} and \textit{in vivo} (12), and make the parasite very vulnerable, because of its partially deficient antioxidant defense system.

It is relevant to highlight that in presence of ascorbic acid for the epimastigote stage a decrease of 10 times in the IC_{50} of B_{12} can be achieved (data not shown). In our \textit{in vivo} model, the additions of ascorbic acid to B_{12} treatment produce an important increase of the antiparasitic activity, since mice treated with both presented a reduction 2 times higher than those which received B_{12} alone on the peak of parasitemia. The fact that an antioxidant enhances the effect of B_{12} would assure that concentrations of B_{12} required to express anti-parasitic activity, do not exceed the maximum concentration that can be achieved in circulation.

Benznidazol is the reference drug used nowadays, however it is unsatisfactory because of its limited efficacy and its toxic side effects such as anorexia, vomiting, peripheral polyneuropathy, and allergic dermopathy. Instead, B_{12} rarely present side effects and is an over the counter drug. As shown in \textbf{Fig 7}, the administration of vitamins together with Bnz,
was able to improve the antiparasitic effect of the Bnz treatment showing its potential as candidates for novel therapeutics modalities for the treatment of Chagas’ disease.

Acknowledgements

FMF, ELM, AB, and MEL hold the post of Scientific Researchers at the Argentine National Research Council (CONICET). ABC, FMF, ELM, and MEL are members of the University of Buenos Aires. ABC is a Post-doctoral Fellow of CONICET.

Funding

This work was supported by the University of Buenos Aires (UBACYT X083 and 20020090200478), and the Argentine National Research Council, CONICET (PIP 5263).

Transparency declarations

None to declare.

References


Legends to Figures

Figure 1: Effect of vitamin B_{12} on the proliferation of *T. cruzi* epimastigotes. The parasites were cultured at 28°C for 7 days with different B_{12} concentrations (0-45 μM) and were counted daily. All other experimental conditions were as indicated in the Materials and Methods section.

Figure 2: Effect of vitamin B_{12} on epimastigotes cultured with different hemin concentrations. *T. cruzi* epimastigotes were grown in presence (45 μM) or absence of B_{12} in culture medium containing different hemin concentrations (0, 10 and 30 μg/ml). The cells were counted daily between day 2 and 6. In the fourth day vitamin B_{12} was added again, at concentration 30 μM or 45 μM (arrow). All other experimental conditions were those described in the Materials and Methods section.

Figure 3: Isobologram describing the interaction between vitamin B_{12} and Bnz against epimastigotes of *T. cruzi*. The fractional inhibitory concentrations (FICs) and the FIC index (FICI) were determined as described in Materials and Methods. FICI>0.5-4.0 indicate no interaction between vitamin B_{12} and Bnz.

Figure 4: Effect of vitamin B_{12} and Bnz on Vero cells viability. Cells were cultured during 24 h in the presence of different concentrations of B_{12} (6 to 2400 μM) and Bnz (3 to 3000 μM). Cell viability was determined by the MTT assay as described in Materials and Methods.

Figure 5: Intracellular oxidative stress during the short-time treatment with vitamin B_{12}. Epimastigotes were treated with B_{12} (15, 30 or 60 μM) during 3, 7 or 24 h. Then, intracellular oxidative stress was evaluated by flow cytometer (n: 20000 cells/analysis). (a) Histograms corresponding to untreated cells (curve 1: control) and treated with B_{12} 30 μM.
during 3, 7 or 24 h (curves 2, 3 and 4, respectively). (b) Time-course of ratio Gm/t/Gmc (see Materials and Methods) for parasites treated with the different concentration of B12.

Figure 6: Effect of vitamin B12 on activities of SOD, Apx and TryR. Experimental conditions were as described in Materials and Methods. The concentration of B12 used was 30 µM. For each enzyme the activity value obtained in the absence of B12 was considered as the control value (100%).

Figure 7: Effect of vitamin B12 and Bnz on the treatment of infected mice. Parasitemia levels (A) and survival curve (B) during the acute infection period in C3H mice infected with 5 x 10^3 bloodstream trypomastigotes of T. cruzi. Mice were treated from day 5 to 10 and 12 to 17 of infection. Parasitemia was determined by counting the number of trypomastigotes in 5 µl of fresh blood collected from the tail every other day. Mortality was recorded every day.
Table 1: Activity of vitamin B$_{12}$ and Bnz on epi-, trypo- and amastigote forms of *T. cruzi*.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Epimastigote IC$_{50}$/3d (μM)</th>
<th>Trypomastigote IC$_{50}$/1d (μM)</th>
<th>Amastigote IC$_{50}$/7d (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B$_{12}$</td>
<td>2.42 ± 0.54</td>
<td>9.46 ± 1.20</td>
<td>10.69 ± 1.50</td>
</tr>
<tr>
<td>Bnz</td>
<td>5.86 ± 0.93</td>
<td>30.26 ± 2.85</td>
<td>4.10 ± 0.55</td>
</tr>
</tbody>
</table>

IC$_{50}$ values were calculated as indicated in Materials and Methods. ND: Not determined.
Table 2: Effect of DTT and ascorbic acid on the anti-*T. cruzi* activity of vitamin B$_{12}$.

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Vitamin B$_{12}$</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.25</td>
<td>5.4 ± 1.5</td>
</tr>
<tr>
<td>DTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>5.9 ± 1.9</td>
</tr>
<tr>
<td>1.0</td>
<td>0</td>
<td>21.3 ± 2.4</td>
</tr>
<tr>
<td>0.5</td>
<td>0.25</td>
<td>23.9 ± 1.8</td>
</tr>
<tr>
<td>1.0</td>
<td>0.25</td>
<td>41.4 ± 2.2</td>
</tr>
<tr>
<td>Vitamin C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>3.7 ± 1.7</td>
</tr>
<tr>
<td>1.0</td>
<td>0</td>
<td>2.4 ± 1.2</td>
</tr>
<tr>
<td>1.5</td>
<td>0</td>
<td>10.3 ± 1.4</td>
</tr>
<tr>
<td>0.5</td>
<td>0.25</td>
<td>18.4 ± 1.2</td>
</tr>
<tr>
<td>1.0</td>
<td>0.25</td>
<td>22.9 ± 1.6</td>
</tr>
<tr>
<td>1.5</td>
<td>0.25</td>
<td>47.7 ± 1.9</td>
</tr>
</tbody>
</table>

0% of inhibition corresponds to parasites cultured in the absence of either B$_{12}$ or antioxidant compound.
(a)

(b)

<table>
<thead>
<tr>
<th>Vitamin B12&lt;sub&gt;b&lt;/sub&gt; (µM)</th>
<th>Ratio (Gm&lt;sub&gt;i&lt;/sub&gt;/Gm&lt;sub&gt;c&lt;/sub&gt;) at different time of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 h</td>
</tr>
<tr>
<td>15</td>
<td>14.15 ± 1.92</td>
</tr>
<tr>
<td>30</td>
<td>11.89 ± 0.53</td>
</tr>
<tr>
<td>60</td>
<td>11.13 ± 0.42</td>
</tr>
</tbody>
</table>