

Evaluation of the Anti-*Trypanosoma cruzi* Effects of the Antipsychotic Drug Levomepromazine

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Received May 22nd, 2012; revised June 24th, 2012; accepted July 15th, 2012

ABSTRACT

Chagas disease, caused by the protozoan *Trypanosoma cruzi*, is a relevant parasitic disease in the Americas. Current chemotherapy relies on Nifurtimox and Benznidazole, which present serious drawbacks, including high toxicity, low efficiency and the emergence of resistant strains. In the present work, the perspectives of levomepromazine, a tricyclic compound belonging to the family of phenothiazines with well-known properties as antipsychotics were evaluated as a potential anti-*T. cruzi* drug. We show that this drug is able to inhibit the proliferation of epimastigotes ($IC_{50} = 0.41 \pm 0.01$ mM) and to interfere with the infection of the host cells ($IC_{50} = 0.34 \pm 0.01$ mM). Interestingly, the treatment with levomepromazine affected the ability of metabolites such as glucose, proline and glutamate to fuel the recovery of epimastigotes after being submitted to metabolic stress. These findings prompt levomepromazine as a promising leader drug to obtain new trypanocidal activities.

Keywords: Chagas Disease; Phenothiazines; Trypanothione Reductase; Amino Acid Metabolism; Chemotherapy

1. Introduction

Trypanosoma cruzi, the causative agent for Chagas disease, a relevant health problem in most American countries, as it is broadly distributed from Mexico to southern Argentina and Chile, where it is endemic, with 10 - 12 million people infected and an estimated 25 million people at risk of acquiring the disease. Two drugs made available almost four decades ago for patients are still the only ones currently in use to treat Chagas disease: Nifurtimox (Nf) and Benznidazole (Bn). Both drugs are highly efficient in the acute phase of the disease; however, their efficiency to treat the chronic phase, when most patients are diagnosed, remains controversial. In addition, it is worth mentioning that other serious drawbacks have been reported for both drugs: their high toxicity, which in some cases forces the interruption of treatment, and evidence of emerging resistant strains. Taken together, these facts constitute major reasons to search for new therapeutic alternatives [1].

Phenothiazines are a family of drugs related to the thiazine class of heterocyclic compounds [2]. Phenothiazines, which consist of a sulphur-containing tricyclic organic compound with the formula $S(C_6H_4)_2NH$, with side chains bound to the middle ring, have been commonly used psychotropic drugs since the 1950s. This family of

compounds became well known and has been prescribed up to the present because of their antipsychotic effects [3]. Their action as dopamine blockers at D_2 receptors and their neuroleptic activity has been well documented in the literature [4-7]. More recently, a large variety of structurally modified versions of these drugs has been introduced, broadening the spectrum of their targets. In this sense, papers published in the last twenty years showed antitumoural, antimicrobial, antiparasitic and anthelmintic activities for phenothiazine derivatives (reviewed in [2]). The biological activities identified for members with different substitutions at different positions, mainly 2 and 10, were particularly interesting to us. For example, it was shown that compounds having a methyl-thio substituent at position 10 and a fluorine substituent at position 2 (triflupromazine) have interesting antimicrobial properties [2]. Variants of these drugs, such as triflupromazine and chlorpromazine, have been active against pathogenic amoebas like *Naegleria fowleri*, *Acanthamoeba culbertsoni* and *A. polyphaga* [8]. Despite unknown mechanisms of action, it was proposed that the drugs have an antagonistic effect on the amoebal calmodulins [9]. It was also proposed that chlorpromazine, a lipophilic variant, could act by modifying the plasma membrane in some way, thereby diminishing the viability of these organisms. Chlorpromazine also showed inhibitory activity on the growth of *Leishmania donovani* promastigotes [10], the fungus *Candida albicans* [11],

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the bacteria *Staphylococcus aureus* [12], *Mycobacterium avium* [13] and *M. tuberculosis* [14]. *Shigella* spp., *Vibrio cholera* and *V. parahaemolyticus* are among the microorganisms reported to be sensitive to trifluoperazine. In the present work, the possible trypanocidal or trypanostatic effect of Levomepromazine (LM), an antipsychotic drug belonging to the family of phenothiazines, was investigated. LM was able to inhibit the growth of epimastigotes in a dose-dependent way, ($IC_{50} = 0.41$ mM) in the range of the doses reported in the literature for most of the tests with these drugs (which approximately range between 0.05 and 0.50 mM). Finally, it is worth mentioning that since the first half of past century, phenothiazines have been used safely in the treatment of human infection by the helminth *Enterobius vermicularis* [15].

In the present work, we report the anti-*T. cruzi* activity of levomepromazine. We also show its ability to synergise the deleterious effects of nutritional stress, metacyclogenesis and host-cell infection.

2. Materials and Methods

2.1. Reagents

D-Glucose, L-proline, L-glutamate, rotenone, antimycin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO, USA). RPMI culture medium and foetal calf serum were obtained from Cultilab (Campinas, SP, Brazil). Levomepromazine (LM) (for chemical structure see **Figure 1**) (Togrel[®]) was purchased from Ivvax (Argentina). All other reagents were from Amresco (Solon, OH, USA).

2.2. Cells and Parasites

The Chinese Hamster Ovary cell line, CHO-K₁, was routinely cultivated in RPMI medium (Gibco BRL) supplemented with 10% heat-inactivated foetal calf serum (FCS), 0.15% (w/v) NaHCO₃, 100 units/mL penicillin and 100 µg/mL streptomycin at 37°C in a humid atmosphere containing 5% CO₂. Epimastigotes of *T. cruzi*, CL strain, clone 14 [16], were maintained in the exponential

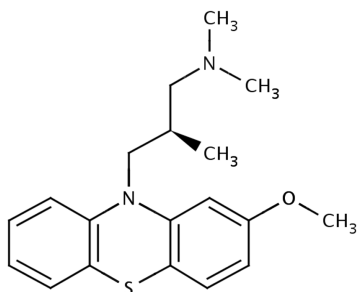


Figure 1. Chemical structure of levomepromazine (LM), a phenothiazine derived compound.

growth phase by subculturing every 48 h in liver infusion-tryptose (LIT) medium supplemented with 10% FCS at 28°C [17]. Metacyclic trypomastigotes were obtained as previously described [18]. Briefly, 20×10^6 epimastigotes in the exponential growth phase were harvested by centrifugation ($5000 \times g$ for 10 min), washed with PBS, and resuspended in TAU medium (190 mM NaCl, 17 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 8 mM phosphate buffer pH 6.0). After 1-h incubation at 28°C, cultures were centrifuged ($5000 \times g$ for 10 min), resuspended in TAU-3AG (TAU supplemented with 10 mM proline, 50 mM L-sodium glutamate, 2 mM L-sodium aspartate and 10 mM D-glucose) and incubated for 96 h at 28°C. Culture-derived trypomastigotes were obtained by infection of CHO-K₁ cells with trypomastigotes, as described previously [19]. Trypomastigotes were collected in the extracellular medium from five days post-infection. In all cases, parasites were counted in a Neubauer chamber.

2.3. Growth Inhibition Assays

Epimastigote growth-inhibition assays were developed as previously described [20]. Briefly, epimastigotes in the exponential growth phase (approximately 5×10^7 cells/mL) were washed three times by centrifugation and resuspended in PBS and then cultured in fresh LIT medium supplemented with or without (controls) different concentrations of LM ranging from 0.2 to 1.1 mM at 28°C. The assays were carried out in 96-well plates by inoculating epimastigotes in 200 µl of medium (2.5×10^6 cells/mL). Cell growth was estimated by absorbance readings at 620 nm every day for ten days. The absorbance was transformed into a cell density value (cells/mL) using a linear calibration equation previously obtained under the same conditions ($R^2 = 0.99551$, $p < 0.05$). The concentration of LM that inhibited 50% of parasite growth (IC_{50}) was determined in the exponential growth phase (fifth day) by adjusting the effect (growth inhibition values) as a function of the drug concentration to a classical sigmoidal equation. As a cell growth inhibition control, growth curves in which 60 µM rotenone and 0.5 µM antimycin were added to the culture medium were run in parallel for all experiments.

2.4. The Effect of LM Growth Inhibition under Stress Conditions

To analyse the combined effect of LM at different pH values and at different temperatures, the growth curves were generated as described above, by adjusting the LIT pH to the desired values or incubating the cultures at 28°C (no stress), 33°C or 37°C. To evaluate the effect of LM combined with nutritional stress conditions, 2×10^7 parasites/mL were washed and resuspended in 0.2 mM

LM, in PBS (as a control) or PBS supplemented with 3 mM glucose, 3 mM proline, or 3 mM glutamate for 48 h in Eppendorf tubes. Subsequently, cell viability was estimated using the MTT assay. To evaluate the combined effect of LM and oxidative stress, 2.5×10^6 parasites/mL in the exponential phase were maintained for 90 min at 28°C in PBS or with 100 μ M H₂O₂ in the presence or absence of 0.2 mM LM. The cells were then collected by centrifugation and resuspended in LIT medium, and after 5 days, the number of cells/mL was determined as described previously [21,22].

2.5. Effect of LM on Mammalian Cell Viability

CHO-K₁ cells (5.0×10^5 cells/mL) were inoculated in 24-well plates in FCS-supplemented RPMI medium, as previously described, in either the absence (control) or presence of increasing concentrations of LM. The cell viability was determined using the MTT assay as previously described, and the IC₅₀ was obtained by fitting the data to a typical dose-response sigmoidal curve [21].

2.6. Effect of LM on Trypomastigote Bursting

CHO-K₁ cells were grown on cover slips (approximately 10^5 cells) and infected with 0.5×10^4 trypomastigotes in RPMI medium supplemented with 10% FCS. After 3 h at 37°C, free trypomastigotes in the medium were removed by washing with PBS, and the infected cells were maintained at 33°C in RPMI medium supplemented with 2% FCS, with or without different concentrations of LM. These concentrations of LM were not toxic to the mammalian cells. The trypomastigotes were collected in the

extracellular medium on the fifth day and counted in a hemocytometer [21].

2.7. Statistical Analysis

All experiments were made at least in triplicates. A one-way ANOVA followed by Dunnet's test was used for statistical analysis. To analyse synergism between two independent treatments, a two-way ANOVA was performed as described previously [23]. A *p* value less than 0.05 was considered statistically significant.

3. Results

3.1. Evaluation of the Trypanocidal Effect of LM

To investigate the effects of LM on *T. cruzi* growth, 2.5×10^6 epimastigotes/mL were cultured in LIT medium supplemented with different concentrations of the drug, ranging from 0.2 to 1.1 mM. The growth of epimastigotes in LIT supplemented with 6 μ M rotenone and 0.5 μ M antimycin as well as non-supplemented LIT were used as positive and negative controls for growth inhibition, respectively. The cultures corresponding to non-supplemented LIT showed typical growth curves reaching the maximum values of cell density (86.0×10^6 cells/mL at day 9) when compared to the treated cultures (which ranged between 69.0 and 2.2×10^6 cells/mL at day 9) (Figure 2). As expected, the rotenone/antimycin-treated cultures did not grow. A dose-response effect was observed for the treated parasites, in which the effect of the drug was significant for all evaluated concentrations of LM on day 5 after treatment, when compared with LIT

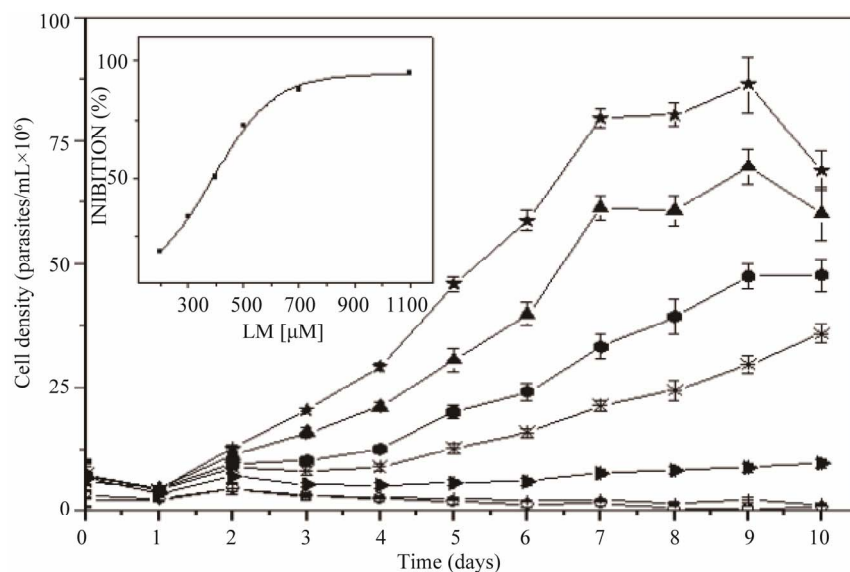


Figure 2. Growth curve of epimastigotes of *Trypanosoma cruzi* treated or not with LM at 28°C and pH 7.5: ★: 0 mM (negative control), 1.1 mM (◻), 700 μ M (◄), 500 μ M (*), 400 μ M (●), and 200 μ M (▲). Positive control for growth inhibition (○) was performed using 0.5 μ M antimycin and 6 μ M rotenone. Inset: dose response curves of epimastigote densities at different LM concentrations on day 4 post treatment.

controls. The IC_{50} value was determined to be 0.41 ± 0.01 mM (**Figure 2**, inset).

3.2. Interaction of LM with Stress Conditions

As mentioned above, *T. cruzi* circulates between two different types of hosts, vertebrates and invertebrates. Within each host type, this parasite goes through different territories, meaning that these organisms are exposed to different physical, physicochemical and metabolic environments throughout their life cycle. Thus, they are always exposed to a variety of natural stress conditions, including nutritional, oxidative, pH and thermal stresses. As a result, we were interested in evaluating the interaction between the treatment of *T. cruzi* epimastigotes and these stress conditions.

3.2.1. Oxidative Stress

To investigate the effect of LM on parasites under oxidative stress, cultures were initially exposed to different concentrations of H_2O_2 as a challenging agent.

Dose-response curves were obtained from these treatments, establishing the IC_{50} as 0.10 mM H_2O_2 . To evaluate the possible interaction between both treatments, the parasites were incubated for 90 min with the IC_{50} concentration of H_2O_2 (0.10 mM) in PBS and further incubated in LIT medium supplemented or not with the IC_{25} concentration of LM (0.25 mM). The combined treatment of LM and H_2O_2 showed a statistically significant effect ($p < 0.05$), measured as a reduction in the capability of parasite to growth at day 5 post-treatment. The percent recovery after treatment for LM-treated cells was 45.00%; for H_2O_2 -treated cells, it was 83.80%; and for the combination of both treatments, it was 95.60% ($p < 0.05$) (**Figure 3(a)**).

3.2.2. Nutritional Stress

To analyse the effect of LM on parasites under nutritional stress, cultures containing 20×10^6 cells/mL were starved by 48 h incubation in PBS or in PBS supplemented with 3 mM proline, glucose or glutamate (PRO, GLC and GLU, respectively). Samples of each culture were concomitantly exposed or not (control) to a treatment of 0.25 mM LM. As previously observed [21,22], the presence of any of the three metabolites contributed to maintain the viability of the cells, compared to those starved in just PBS (**Figure 3(b)**). Each culture treated with LM showed a significant diminution of viability with respect to its corresponding non-LM-treated pair, showing that LM interferes with the ability of proline, glutamate and glucose to extend the survival of parasites under nutritional stress ($p < 0.05$ for all paired comparisons). The combination of nutrients starvation and LM treatment resulted in a synergistic diminution of the vi-

ability relative to the results observed in the presence of any tested metabolite.

3.2.3. Thermal Stress

The viability of epimastigotes exposed to the simultaneous treatment of LM and thermal stress was also evaluated. For thermal stress, three temperatures were chosen: 28°C, the optimal temperature of growth for the insect vector stage; 37°C, the temperature of the mammalian host; and 33°C, the optimal temperature for the progression of *in vitro* infection of mammalian cells [19]. The non-treated cultures behaved as previously observed, with the maximum growth rate and maximum stationary-phase cell densities achieved at 33°C rather than 28°C (**Figure 3(c)**). As previously observed, both the growth rate and stationary-phase cell density were the lowest at 37°C; however, these values were in the range of the control (28°C). If LM interacts with mechanisms related to heat-shock resistance, then a significant change in the IC_{50} values at different temperatures would be expected. However, the obtained results did not show significant interactions between LM and thermal stress.

3.2.4. pH Stress

To evaluate the possible interaction of LM treatment with different pH conditions, the parasites were initially incubated in LIT with the pH adjusted to different values, and the cell growth at day 5 was determined. Parasites maintained at pH = 7.5 grew at rates comparable to those of the controls (control curve **Figure 1**), while the most extreme acidic pH evaluated significantly decreased the cell density achieved in these conditions. Surprisingly, parasites grown in slightly acidic conditions (pH = 6.5) showed the maximum growth inhibition (**Figure 3(d)**). Interestingly, when cultures grown in these conditions were subjected to the IC_{25} of LM (0.25 mM), no significant interaction was found between LM and acidic pH. In fact, an additive effect is clearly observed for each condition, showing that this compound does not interfere with the resistance to this variable.

3.3. The Effect of LM on Differentiation to Infective Metacyclic Trypomastigotes

One relevant process involving environmental sensing in terms of nutrient availability is the differentiation from the non-infective epimastigote to the infective trypomastigotes (a process called metacyclogenesis). Metacyclogenesis begins after the cells are exposed to a nutritional stress, which naturally occurs in the terminal portion of the digestive tube of the invertebrate host. Because LM treatment interacts synergistically with the effect of nutritional stress, we concluded that LM could also be interfering with metacyclogenesis. Epimastigotes were incubated in TAU-3AG differentiation medium

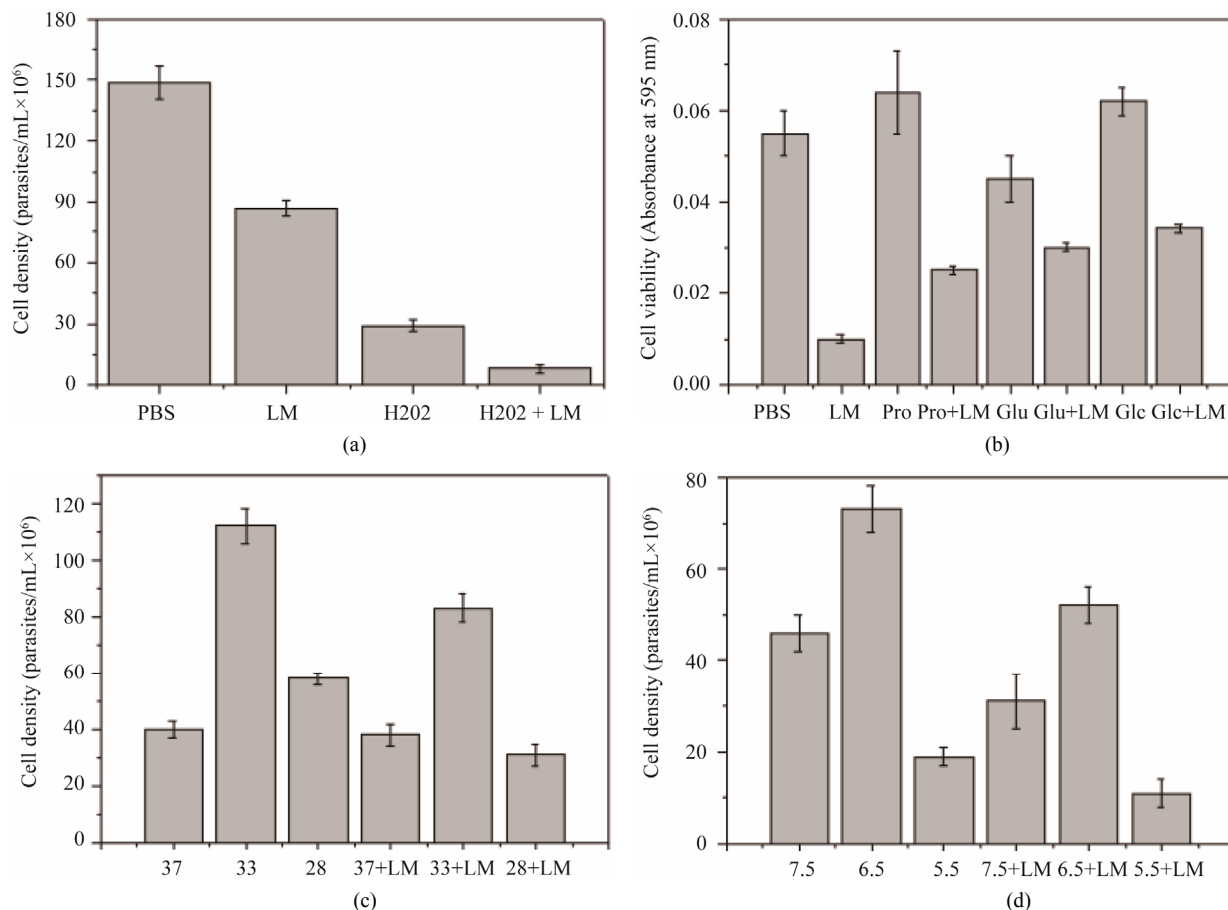


Figure 3. Response of epimastigotes at different stress conditions and LM treatment. (a) The parasites were submitted to oxidative stress by incubation in the presence of 0.10 mM H₂O₂ and the addition or not of drug (0.25 mM LM). The effect of treatments and controls were measured by growing the parasites in LIT for five days and quantifying the cell density; (b) The parasite viability was measured using the MTT assay after nutritional stresses performed by 72 h incubation in PBS, PRO, GLU or GLC, combined or not with the drug treatment (0.25 mM LM); (c) The parasite density was measured at the 4th day of growth, submitted or not to thermal stress (33°C and 37°C) and drug treatment (0.25 mM LM); (d) The parasite density was measured at the 4th day of growth, submitted or not to pH stress (pH 5.5 or 6.5) and drug treatment (0.25 mM LM).

supplemented with or without (control) 0.41 mM LM. The number of metacyclic forms was counted at 48, 72 and 96 h post-treatment. Because the treatment was performed with the IC₅₀, in the absence of any type of interference with this differentiation process, a 50% decrease in the quantity of metacyclic forms for the treated cells with respect to controls would be expected due to 50% death. Interestingly, metacyclics were inhibited by 71.47%, 80.00% and 88.79% at 48, 72 and 96 h post-treatment, respectively (Figure 4), clearly showing that LM diminishes the ability of epimastigotes to differentiate into metacyclic trypomastigotes.

3.4. Cytotoxicity and Effect of LM on Trypomastigotes Bursting from Infected Host Cells

To evaluate the effects of LM on the treatment of infected cells, the toxicity of LM on CHO-K₁ cells was

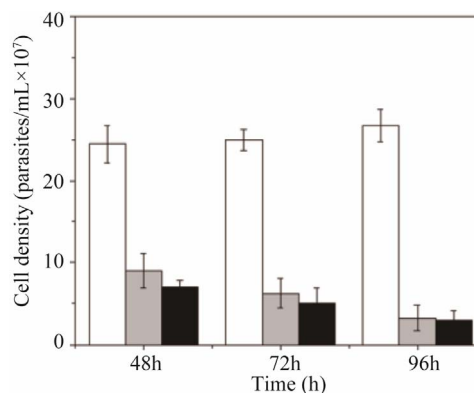


Figure 4. The effect of LM on parasites differentiation. Epimastigotes were incubated for different times (48, 72 or 96 h) in TAU-3AG differentiation medium for metacyclogenesis in the presence of 0 (Control) mM of LM (white bars), 0.3 mM of LM (grey bars), or 0.4 mM of LM (black bars). Metacyclic forms were counted by microscopical observation in a Neubauer chamber.

initially evaluated. The cells were incubated for 48 h in culture medium supplemented with or without (control) LM at concentrations ranging between 0.2 and 1.2 mM. The IC_{50} was calculated to be 0.86 mM (Figure 5). Despite this value being close to the IC_{50} obtained for *T. cruzi* epimastigotes, the effect of LM on the trypomastigote bursting from the infected cells was evaluated. Cells (1×10^6 /well) were infected with 0.5×10^6 culture-derived trypomastigotes and incubated with medium supplemented with or without (control) LM in a range of concentrations between 0.1 and 0.6 mM. Interestingly, the treatment with 0.5 mM LM reduced the trypomastigote bursting by more than 96%, resulting in an IC_{50} of 0.34 mM (Figure 6).

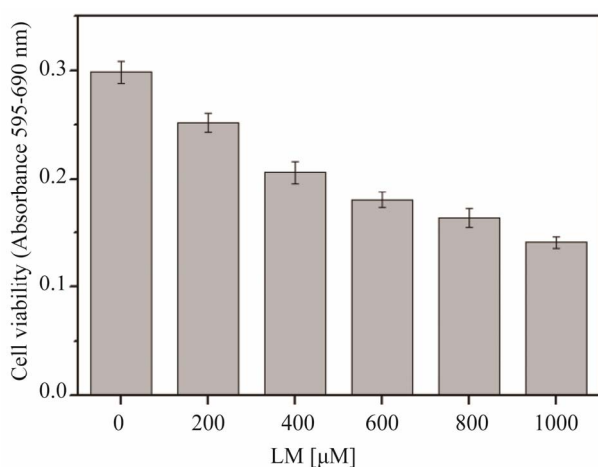


Figure 5. Evaluation of the cytotoxic effect of LM on CHO-K₁ cells. CHO-K₁ cells were challenged with different concentrations of LM, and the cell viability was measured using the MTT assay.

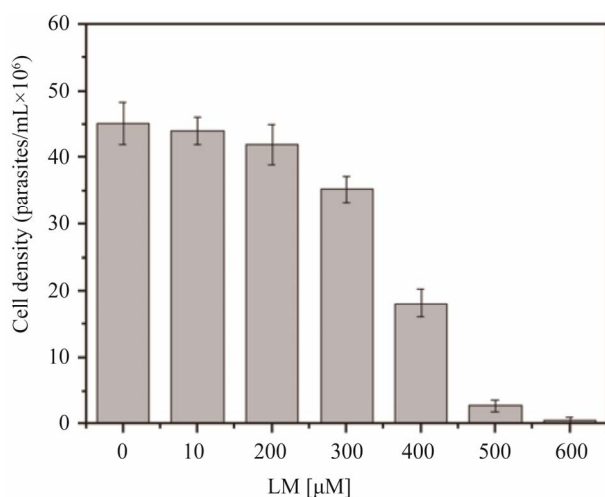


Figure 6. The effect on the trypomastigotes bursting of the treatment with LM of infected cells. The trypomastigotes that bursted on the 7th day post-infection in cell cultures treated or not with different concentrations of LM were counted.

4. Discussion

As mentioned, phenothiazines are compounds that belong to a large family of molecules with increasing prospects for neuropathology therapy, cancer, immunomodulation and infections [2,3]. As *T. cruzi* circulates throughout different environments (different regions of the digestive tube in the insect vector, or intra- and extracellular medium in the mammalian host), it is naturally exposed to several stresses in its life cycle [24]. Among these natural stresses, oxidative, nutritional, thermal and pH stress are relevant. Several vital mechanisms related to the ability of *T. cruzi* to cope with these conditions have been described [25]. The interference of LM with such mechanisms could be relevant to prospect an *in vivo* study of LM by itself or in combination with other drugs. No interference of LM with oxidative, thermal or pH stress was found in the present study. However, LM interfered with the ability of the parasites to recover from nutritional stress. The ability of proline, glutamate, aspartate and glucose to delay the effect of parasite starvation was previously shown [22]. Interestingly, LM interfered with this ability when each of the aforementioned metabolites was evaluated. This set of results strongly suggests that LM is interfering with *T. cruzi* metabolism in a way that affects its ability to use the tested carbon sources.

When the parasites were simultaneously submitted to LM and oxidative, pH and thermal stress, no interactions were observed. It is interesting the fact that, in spite of being considered the optimal temperature as being 28°C, our experiments show that in our system, the optimal temperature was 33°C. This fact was previously observed by us and described as being probably strain dependant [21,22]. Starvation leading to nutritional stress occurs when the parasite changes its environment throughout its life cycle [26]. Particularly, it is well known that nutrient starvation occurs in the transit from the medium to the terminal portion of the insect gut [18]. In the rectum, some nutrients are available due to their back-flow to the digestive tube from the hemolymph through the Malpighian tubules to the rectum. The most relevant nutrients are precisely proline, glutamate and aspartate [18], which are able to recover the intracellular ATP levels that fuel metacyclogenesis [27]. Our results showed that the ability of these nutrients to keep the epimastigotes viable was abolished by treatment with LM. This finding supported the hypothesis that LM could also interfere with metacyclogenesis when induced by these metabolic substrates. This rationale led us to evaluate the metacyclogenesis gated by TAU-3AG in the presence or absence of LM. The results obtained confirmed that metacyclogenesis was diminished by half in these conditions; thereby supporting the idea that LM interferes with the metabolic pathways that energetically support the intense transformation experienced by these cells through meta-

cyclogenesis.

Finally, the effect of LM on trypomastigote burst was also evaluated. Our results showed that the treatment of infected cells with 0.5 mM LM resulted in more than 90% inhibition of trypomastigote bursting. The treatment of *T. cruzi* with another phenothiazine derived drug, thioridazine (usually prescribed as a neuroleptic drug), at low doses seemed to be promising for epimastigotes (IC₅₀ = 5 µM). However, trypomastigotes were less sensitive to the drug, showing an IC₅₀ of 0.5 mM [28]. In addition, the treatment of mice with thioridazine partially prevented the development of parasitemia; however, some cardiac damage was not prevented, even in the absence of the evidence of amastigote nests [29].

Collectively, our data support the idea that the metabolic steps involved in the oxidation of glucose, proline, aspartate and glutamate are involved in the antiparasitic activity of LM. Further experiments will be conducted to define the molecular targets involved in this antiparasitic activity, which will, in turn, lead to the optimisation of more efficient LM-derived drugs.

5. Acknowledgements

The authors are deeply acknowledged to Dr. Laura Tanga for helpful discussions and interesting suggestions. Financial Support: this work was supported by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP grant #11/50631-1 to AMS), Instituto Nacional de Biologia Estrutural e Química Medicinal em Doenças Infecciosas (INBEQMeDI), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq grant # 470272/2011-2 to AMS).

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