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Effect of mesoionic 4-phenyl-5-(cinnamoyl)-1,3,4-thiadiazolium-2-phenylamine chloride derivative salts on the activities of the nitric oxide synthase and arginase of *Leishmania amazonensis*

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Abstract

L-arginine is involved in the production of both nitric oxide (NO), mediated by nitric oxide synthase (NOS) and L-ornithine, by arginase activity. It is generally accepted that NO regulation occurs mainly at the transcriptional level of NOS. In a previous work we purported that there is evidence that *Leishmania* sp. can produce NO from L-arginine. An arginase activity in its gene sequence has also been reported in *Leishmania* parasites. In a search for intracellular targets as potential antileishmanicidal agents, such as the L-arginine metabolism, we used 1,3,4-thiadiazolium mesoionic compounds, that have been demonstrated to be cytotoxic to the *Leishmania amazonensis,* when compared to Pentamidine isethionate as a reference drug. Parasites were assayed in absence/presence of 4′- and 3′- methoxy mesoionic derivatives in order to verify the effect on NO production and arginase activity in *L. amazonensis.* The results indicated that the drugs reduce from 70 to 90% of the NO production by the parasite and act on a soluble nitric oxide synthase purified from *L. amazonensis* promastigotes and axenic amastigotes.

Keywords: Leishmania amazonensis*, mesionic compounds, phenyl-5-(cinnamoyl)-1,3,4-thiadiazolium-2-phenylamine, nitric oxide, nitric oxide synthase, arginase*

Introduction

Leishmania parasites are small and intriguing organisms that cause Leishmaniasis (cutaneous, mucocutaneous and visceral) in tropical and subtropical regions of 88 countries [1]. Thus, more efforts have been concentrated on the further identification of constituent intracellular molecules and the possible antileishmanicidal drug targets. Arginase hydrolyzes L-arginine into L-ornithine and urea. L-ornithine favors parasite growth and it is a precursor for the synthesis of L-glutamine, L-proline and polyamines via the ornithine decarboxylase (ODC) pathway. Polyamines have multiple roles in stabilizing nucleic acid and membranes, as well as regulating cell growth and differentiation [1,2].

Recent results from our laboratory reported NO production by *Leishmania* sp. and the participation of the NO-L. *amazonensis* pathway in the host/parasite interaction [3–5]. Although the macrophages triggered their defense mechanism to neutralize the parasite, there are evidences that the NO pathway from *L. amazonensis* participates in the parasite-host interaction, and there is a correlation between NO production and the amount of metacyclic forms in the culture of infective forms [4–6]. Additionally, recent results also demonstrated that *L. amazonensis* axenic amastigotes produce NO [7], as well as the fact that the NOS activity is evident in this evolutive form, suggesting that both the NOS pathway and the arginase are cross talking during the signaling involving host cell-parasite interaction [3–8].

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Arginase is a binuclear manganese metalloenzyme that catalyses the hydrolysis of L-arginine into L-ornithine and urea, and this activity has been implicated in the regulation of the NO synthesis, modulation of the availability of L-arginine and consequently being linked to the cytotoxic processes in immunological mechanisms of protection against infectious diseases [9,10]. The activity of arginase in parasitic protozoa of the Trypanosomatidae family was shown to be genus specific and has been used as an identification and classification tool. *Leishmania* is one genus in that family in which arginase activity is detectable and is considered to play a role in ornithine production [10].

The identification of parasite factors that induce arginase, as well as the membrane receptors and signaling involved, will make it possible to target the best way to inhibit the modulation NOS/arginase pathway. Thus, arginase inhibition could be useful in some tissues, in which NO production is required, but should be avoided in other tissues, where NO production has to be down regulated. Moreover, a great care must be taken to avoid blocking host urea formation. Therefore, besides the use of arginase inhibitors, the modulation of arginase transcription could represent a new field of investigation [11].

As a part of our research program on experimental chemotherapy against Leishmaniasis, we decided to evaluate the effect of two salts of mesoionic derivatives on the NOS/arginase pathways of the parasite. Mesoionic salts have provided numerous compounds with antifungal, antibacterial and antitumor (against Ehrlich carcinoma and Sarcoma) activities [12,13]. These compounds possess structural features, which have been of considerable interest to medicinal chemistry. They are characterized as containing a five or six-membered heterocyclic aromatic ring that has a partial positive charge which is balanced by a corresponding negative charge on an exocyclic atom or group and must be planar or nearly so as well as possessing considerable resonance energy. Their potential value as biologically active substances is found in the planar aromatic character (Figure 1). The association of these characteristics with the little polyhetero-atomic system suggests a high probability of strong interactions with the biomolecules, such as proteins [13]. In contrast with sydnones, sydnonimines and oxatriazol (Figure 2a, 2b, 2c), 4-phenyl-5-(cinnamoyl)-1,3,4-thiadiazolium mesoionic derivative salts are not NO donors (Figure 2d).

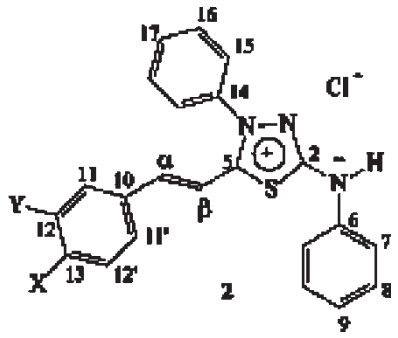

Figure 1. Chemical structure of the mesoionic salt derivatives, 4-phenyl-5-(3′-Y- or 4′-X-cinnamoyl)-1,3,4-thiadiazolium-2-phenylamine chlorides. The numeration of structure is only for chemical shifts attribution.

Materials and methods

Reagents

Benzamidine, trypsin inhibitor, penicillin G, KCl, leupeptin, L-glutamine, Schneider's Insect Medium, $MgCl_2$, phenylmethylsulfonyl fluoride (PMSF), N-1-naphtylethylenediamine, phosphoric acid, polyethyleneglycol, sulfanilamide, sucrose, Tris–HCl, dithiotreitol (DTT), aprotinin, L-arginine, NADH, NADP, NADPH, EGTA-AM (acethoxy methyl ethylene glycol-bis [β-aminoethyl ether]-N,N,N′,N′-tetra acetic acid), L-NAME (N^{ω}-nitro-L-arginine methyl esther), (6R)-5,6,7,8-tetrahydrobiopterin (H_4B), 2′-5′-ADP agarose, RPMI and rabbit IgG-anti nNOS (N7280/rat brain origin) were from Sigma Chemical Co., St. Louis, MO (USA). Glycerol, fluorescein isothiocyanate and formaldehyde were from Bio Rad (USA). Fetal calf serum (FCS) was from Gibco BRL (USA). Electrophoresis was performed using Phast-System (Pharmacia LKB Biotechnology Inc.).

Parasites

Leishmania amazonensis (MHOM/BR/77/LTB0016 strain), infective promastigotes (IP) and axenic amastigotes (AA) were studied comparatively. IP (4^{th} passage) had a high percentage (~ 73%) of metacyclic forms, evaluated through complement lysis test. Promastigotes were maintained in Schneider's Insect

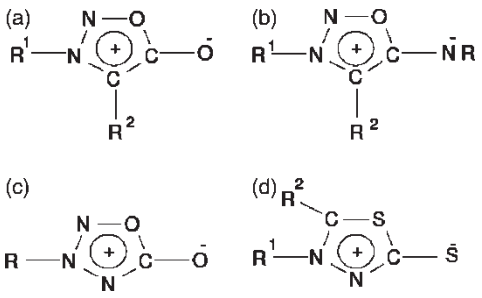

Figure 2. Mesoionic compounds (sydnone and thiadiazolium). 2a = sydnones (1,2,3-oxadiazolium-5-olate); 2b = sydnonimines (1,2,3-oxadiazolium-5-aminide); 2c = oxatriazoles (1,2,3,4 oxatriazoles-5-olate); 2d = 1,3,4- thiadiazolium mesoionic (1,3,4-thiadiazolium-2-thiolate).

Medium supplemented with 10% of heat inactivated FCS at 26°C and pH 7.2.AA were also cultivated in the same medium, but with 20% FCS at 32°C and pH 5.5 [15].

Drug test

Both developmental stages of *L. amazonensis* were added with the corresponding LD_{50}/24 h of mesoionic salts (test compounds) and pentamidine isethionate (reference drug substance/$LD_{50} = 0.46\mu M$ for promastigotes and 118μM for amastigotes) [16]. 1,3,4,-thiadiazolium mesoionic compounds were obtained from the Department of Chemistry, Federal Rural University of Rio de Janeiro (UFRRJ). The more active mesoionic compounds were tested: 4-phenyl-5-(4′-R- or 3′-R-cinnamoyl)-1,3,4-thiadiazolium-2-phenylamine chlorides, were $R =$ methoxy, in the following concentrations: 1.7 mM and 2.4 mM, for promastigotes and 167.7 mM and 120.8 mM for amastigotes, respectively. Infective promastigotes and axenic amastigotes (4×10^6) were cultured in absence/presence of the compounds. The mesoionic compounds were added to parasite cultures solubilized in DMSO (the highest concentration used was 1.6%) v/v, not hazardous to the parasites) [16].

Nitrite determination

After 24 h of culture, parasites were counted in a Neubauer's chamber centrifuged at 1,500 g for 10 min and the supernatants were collected. Nitrite, a stable oxidation product of NO, was measured spectrophotometricaly by adding Griess reagent (0.1% N-1-naphtylethylenediamine in 5% phosphoric acid and 1% sulfanilamide) to the same volume of parasite (IP/AA) culture supernatants. After 10–15 min at room temperature, the absorbance of the chromophore was measured at 540 nm (JEWAY 6405 Spectrophotometer, England). Nitrite concentrations were estimated by comparison with a standard curve prepared with sodium nitrite in Schneider's medium with FBS [4,6,12,17].

Isolation of NOS from L. amazonensis *promastigotes and axenic amastigotes*

The purification protocol of NOS was the same used by Basu et al. [18], with some modifications, as follows: cell-free extract was prepared from IP and from AA by freeze-thawing the cell suspension (5×10^9 cells mL^{-1}) 3–5 times and sonicating 5 × 45 s over ice in 0.25 mol/L sucrose containing 5 mM KCl. The contents were centrifuged at 10,000 × g for 30 min and the supernatant was adjusted to antiproteolytic buffer containing 0.1 mM PMSF (phenylmethylsulfonyl fluoride), 0.01% (p/v) leupeptin, 0.2 mg/mL trypsin inhibitor and 1 mM benzamidine to a final volume of 5 mL. The crude soluble extract was centrifuged at 100,000 × g for 60 min at 4°C and the supernatant (1.3 mg/mL protein) was applied to a column (8.5 × 1.5 cm) packed with 2′,5′- ADP agarose equilibrated with 10 mM Tris–HCl, pH 7.5 containing 1 mM DTT (dithiotreitol), 1 mM EDTA, 0.5 mM PMSF, 25 units/mL aprotinin and 0.5 mM L-arginine (buffer A). The column was then washed successively with 20 mL of buffer A, 20 mL of buffer A containing 0.5 mM NADH, 20 mL of buffer A contaning 0.5 mM NADP and 20 mL of buffer A. The enzyme was eluted with 10 mL of buffer A containing 10 mM NADPH, 3 μM H_4B and 10% (v/v) glycerol and concentrated to 2 mL using a cellulose semipermeable membrane/polyethyleneglycol 400 (PEG 400) system. The protein concentration (240 μg/mL) was estimated spectrophotometricaly (260–280 nm) and the sample was utilized to evaluate the pureness, through SDS-PAGE [19].

SDS-PAGE

Electrophoresis (Polyacrylamide gel containing sodium dodecyl sulphate/ SDS-PAGE) was performed using Phast-System on a 10–15% gel according to the manufacturer's directions. The gels were stained with silver nitrate. Molecular weight standards (Bio-Rad) were: myosin (200 kDa), β-galactosidase (116.2 kDa), rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa) and egg white ovoalbumin (42.6 kDa).

*NOS-*L. amazonensis *activity*

The NOS activity purified from IP and AA was determined by measuring the reduction in the absorbance at 340 nm for 10 min continuously, once the NADPH was consumed during the conversion of L-arginine to L-citrulline by NOS [20]. Briefly, The complete enzyme reaction mixture contained 50 mM potassium phosphate buffer (pH 7.4), 1 mM $CaCl_2$, 0.1 mM NADPH, 80 μM H_4B, 10 μM FAD, 10 μM FMN, 0.1 mM L-arginine and 2 μg enzyme in a final volume of 1 mL, at 25°C. Alternatively, 4-phenyl-5-(3′ or 4′-methoxy-cinnamoyl)-1,3,4-thiadiazolium-2-phenylamine chlorides were used in the reaction mixture in the follows concentrations: 1.7 mM and 2.4 mM, for promastigotes and 167.7 mM and 120.8 mM for amastigotes, respectively. Pentamidine isethionate was used in the reaction mixture as a drug control using the corresponding LD_{50} [16]. The control group had all the components of the reaction mixture, except the drugs, and the blank had all the components, except NADPH and the drugs [6,19].

Arginase-L. amazonensis *activity*

Arginase activity from *L. amazonensis* promastigotes and axenic amastigotes treated for 24 h with corresponding LD_{50} of 4-phenyl-5-(3′or 4′-methoxy-cinnamoyl)-1,3,4-thiadiazolium-2-phenylamine chlorides was verified as previously described [21], with some modifications. Briefly, 10^6 cells previously washed with a solution of 0.25 M sucrose and 5 mM KCl were added to 0.5 mL of 0.1% Triton X-100 in an antiproteolytic buffer containing 0.1 mM phenylmethylsulfonyl fluoride, 0.01% w/v leupeptin, 0.2 mg/mL trypsin inhibitor and 1 mM benzamidine in 0.25 M sucrose and 5 mM KCl. The mixture was then stirred for 30 min at room temperature. After the cells were lysed, 0.5 mL of 25 mM Tris–HCl plus 5 mM of $MnCl_2$, pH 7.4 were added and the enzyme was activated for 10 min at 56°C. Arginine hydrolysis was initiated by the addition of 25 μL of 0.5 M L-arginine, pH 9.7 to a 25 μL aliquot of the previously activated lysate. Incubation was performed at 37°C for 60 min and the reaction was stopped by the addition of 400 μL of an acid mixture containing H_2SO_4, H_3PO_4, and H_2O (1:3:7). The urea formed was colorimetricaly quantified at 540 nm after addition of 25 μL of 9% α-isonitrosopropienophenone (dissolved in 100% ethanol) and heating at 100°C for 45 min. After 10 min in the dark, the optical density was determined in a spectrophotometer using 200 μL aliquots in a non-sterile micro-culture plate. A calibration curve was prepared with increasing amounts of urea between 1.5 and 30 μg/mL.

Data analysis

Three independent experiments were performed and the data obtained with different treatments was analyzed statistically using 1-way ANOVA and Students *t*-test ($p < 0.05$) and expressed as the mean ± standard deviation.

Results and discussion

The NO released by *L. amazonensis* IP (stationary phase) and AA was measured by the nitrite concentration in the culture supernatants, using Griess reagent. The results showed that 4-phenyl-5-(3′- or 4′-methoxy-cinnamoyl)-1,3,4-thiadiazolium-2-phenylamine chlorides reduce the NO production in IP cultures (Figure 3a). The reduction was of about 80% (R = 3′-methoxy-cinnamoyl, $p = 0.002$) and 70% (R = 4′-methoxy-cinnamoyl, $p = 0.0011$). Pentamidine isethionate (reference drug) was able to inhibit only 2% of the NO production compared to control ($p = 1{,}45$). When the assay was made using AA cultures, 3′-methoxy-cinnamoyl inhibited the NO production, showing the same profile observed in IP cultures ($p = 0.0034$), but the 4′-methoxy-cinnamoyl compound was less effective in AA (inhibition of 25%, $p = 0.019$). In AA cultures, pentamidine isethionate inhibited the NO production by about 30% ($p = 0.03$) (Figure 3b).

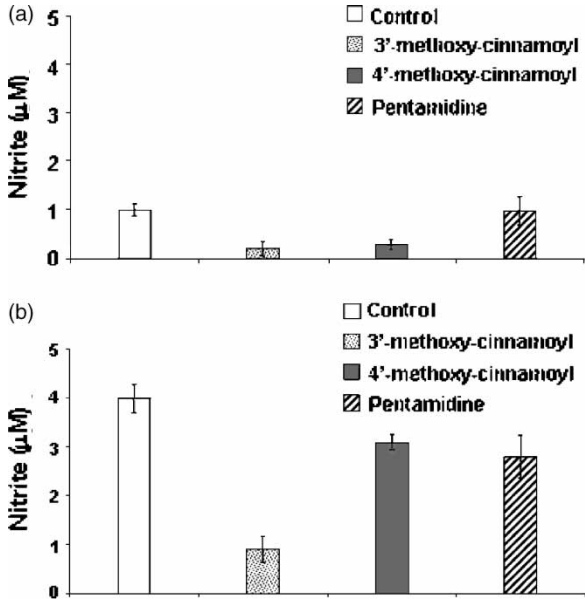

Figure 3. Comparative levels of NO released as nitrite in the supernatant of infective promastigotes/IP (a) and axenic amastigotes/AA (b) cultures of *L. amazonensis* in presence of 4-phenyl-5-cinnamoyl-1,3,4-thiadiazolium-2-phenylamine chlorides (3′- or 4′-methoxy), where 3′- was more active than 4′-compoundand Pentamidine isethionate in axenic amastigotes.

Since the NO production could be intervening directly or not with the activity of the NOS, it was verified that, after the NOS purification from IP

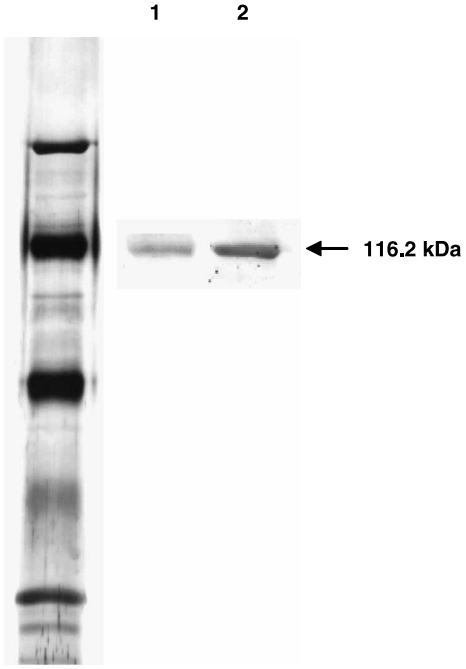

Figure 4. SDS-PAGE of NOS molecule purified from *L. amazonensis* promastigotes (line 1) and axenic amastigotes (line 2).

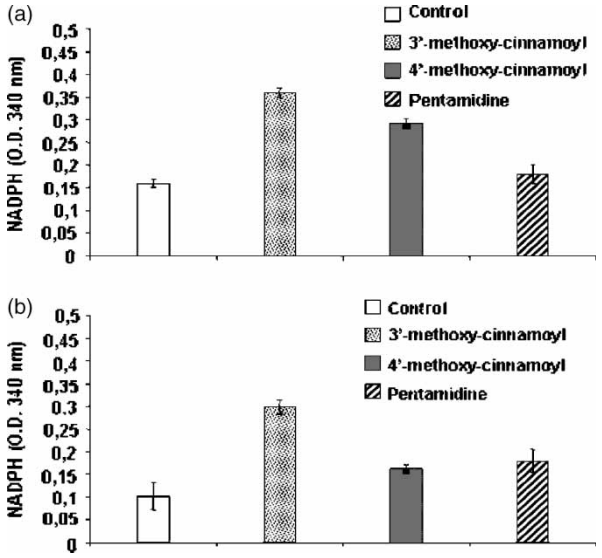

Figure 5. Evaluation of the activity of NOS purified from *L. amazonensis* promastigotes/IP (a) and axenic amastigotes/AA (b), according to the consumption of NADPH, in presence/absence of mesoionic compounds. Data represent mean ± S.D. of three independent determinations.

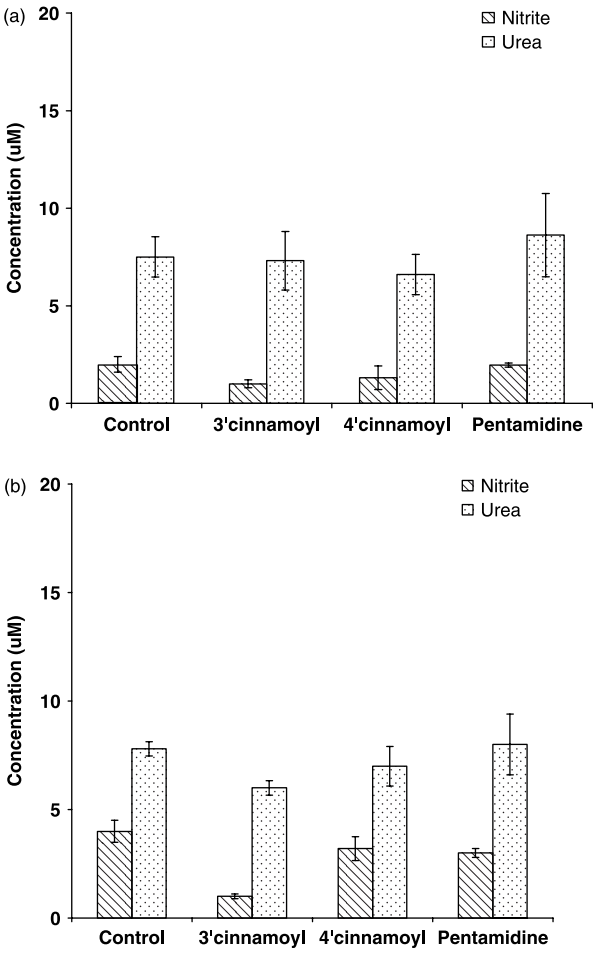

Figure 6. Arginase activity from *L. amazonensis* promastigotes/IP (a) and axenic amastigotes/AA (b), according to urea production, where 3′-methoxy cinnamoyl was able to decrease the arginase activity in amastigotes.

(Figure 4/line 1) and AA (Figure 4/line2), through 2′,5′-ADP-agarose column, the activity assay of the enzyme, through NADPH consumption, showed that the mesoionic compounds act directly on purified enzymes (Figure 5). This data indicates the significant reduction in consumption from about 2.33 (3′-methoxy/p = 0.003) to 1.9 (4′-methoxy/p = 0.025) fold in NOS-purified from IP cultures (Figure 5a) and in NOS-purified from AA, the reduction of the cofactor consumption was from about 2.9 (3′-methoxy/p = 0.0025) to 1.6 (4′-methoxy/p = 0.051) fold (Figure 5b) compared to the control (without drugs), while Pentamidine inhibited 1.8 fold (p = 0.05).

The effect of 3′ or 4′-methoxy-cinnamoyl mesoionic salts on arginase activity is showed in Figure 6. There is no significant reduction of arginase activity in IP (Figure 6a), but in AA, 3′-methoxy-cinnamoyl significantly inhibited arginase activity and this reduction was about 30% (p = 0.003) (Figure 6b).

Mesoionic systems have provided numerous compounds with useful and wide-ranging biological activities. These compounds have well separated regions of positive and negative charges. This association with the polyhetero-atomic system suggested a high probability of strong interaction with biomolecules. Searching for intracellular targets of anti-*Leishmania* drugs, we tested some mesoionic derivatives against NOS and arginase pathways of *L. amazonensis*, which seems to be important during the parasite-host cell interaction [1,4,5,18,21,22].

NO synthesis has always been observed in mammals [23] and invertebrates [24], through NOS. The first demonstration of the existence of the NO pathway in Trypanosomatids was made in 1995 by Paveto and collaborators [25], in *Trypanosoma cruzi*. The dependence of cofactors, as well as the susceptibility of the inhibitors/activators, demonstrated the similarity of the enzyme with the one which participates in the neuronal transduction in mammals. The NOS system was also evidenced in *L. donovani*, but the biological significance, as well as the relation with the pathway existing in the macrophage (induced and harmful for these microorganisms), is still unknown [18].

Considering the importance of the cellular signaling pathways involving the host cells and parasites of the *Leishmania* genus, there are few studies in the literature related to the importance of the arginase and NOS activity in the parasite. In our researches of experimental chemotherapy to treat Leishmaniasis, we evaluated the effect of the more active 4-phenyl-5-cinnamoyl-1,3,4-thiadiazolium-2-phenylamine chlorides (3′- or 4′-methoxy) which showed a significative effect in the reduction of the NO production in promastigotes and axenic amastigotes cultures. The inhibition was evident through NADPH consumption

by NOS purified from both evolutive forms of the parasite (that presented the cNOS form of the enzyme) [6,7]. No significant effect on the arginase activity was verified in IP, but the 3′-methoxy cinnamoyl compound was able to inhibit this enzyme activity.

Many mesoionic derivatives possess useful biological activity [12,13,26,27]. The structural characteristics of mesoionic compounds, which contain distinct regions of positive and negative charges associated with a poly-heteroatomic system, enable them to cross cellular membranes and interact strongly with biomolecules. Other mesoionic compounds (sydnones, sydnonimines and oxatriazol) are, *in vivo*, NO donors, therefore possessing oxygen instead of sulphur as the heteroatomic system of the mesoionic ring [14,28]. In contrast, the thiadiazolium compounds used in this work are not NO donors. This characteristic increases the positive charge on the mesoionic ring and allows extensive conjugation of the side-chain with the exocyclic moiety. This seems to be important for the quantification of the anti-*Leishmania* activity in these compounds. Furthermore, the recognition sites on NOS, arginase and axenic amastigotes provide multiple ways for the regulation of the NO and polyamines levels as well as the cross-talk between second messenger systems, making, this hypothesis worthy to be investigated in *Leishmania* sp.

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