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Research Brief

In vitro evaluation of 4-phenyl-5-(4′-X-phenyl)-1,3,4-thiadiazolium-2-phenylaminide chlorides and 3[N-4′-X-phenyl]-1,2,3-oxadiazolium-5-olate derivatives on nitric oxide synthase and arginase activities of *Leishmania amazonensis*

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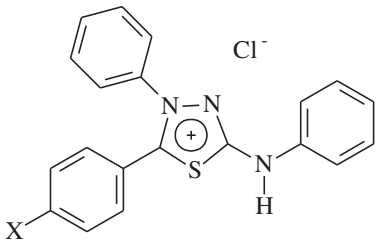
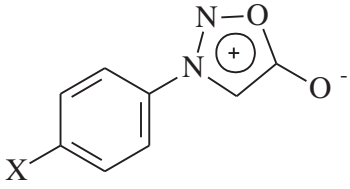
HIGHLIGHTS

- Mesoionic compounds were evaluated against *Leishmania amazonensis.*
- Mechanism of action focused the activities of nitric oxide synthase and arginase.
- All derivatives tested were able to inhibit the parasite nitric oxide synthase.
- None of the compounds was able to inhibit the arginase activity of axenic amastigotes.
- Those compounds were able to inhibited arginase activity in promastigotes.

GRAPHICAL ABSTRACT



X = H (**MI-H**), NO_2 (**MI-NO$_2$**) and OCH_3 (**MI-OCH$_3$**)



X = H (SID-H), NO2 (SID-NO2) and OCH3 (SID-OCH3)

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ABSTRACT

Leishmaniasis is a spectrum of infectious diseases caused by Leishmania protozoan parasites. The purpose of this study was to perform, in vitro, a comparative analysis of the activity amastigotes. Results showed excellent efficacy of all compounds against axenic amastigotes, compared to pentamidine isethionate, the reference drug used. The cytotoxic effect of these mesoionic compounds of six mesoionic compounds (three 1,3,4-thiadiazolium-2-aminide and three 1,2,3-oxadiazolium-5-olate class compounds) was evaluated in mouse peritoneal macrophages using MTT assay, low toxicity (~10%) for these mammalian cells being observed. In an attempt to define a potential drug target, the activities of nitric oxide synthase (NOS) and arginase of the parasites treated with the mesoionic derivatives were evaluated. NOS was purified from a cell-free extract of infective promastigotes and axenic amastigotes and all derivatives tested were able to inhibit the enzyme as monitored by the decrease of NADPH consumption. Arginase activity from both stages of the parasite was measured using urea production and none of the compounds inhibited the enzyme activity of axenic amastigotes. On the

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other hand, when tested with promastigotes, those compounds without and substituents inhibited arginase activity.

1. Introduction

Leishmaniasis is a spectrum of diseases caused by protozoa parasites of the *Leishmania* genus which are transmitted by sandflies (Singh and Sivakumar, 2004). There are over 17 species of *Leishmania* known to be infective to humans. These have been characterized on the basis of biochemical and molecular differences which provide a structure for phylogenetic analysis and improved methods of species identification and diagnosis (Herwaldt, 1999). Today there are 12 million humans infected, mostly distributed in tropical and subtropical countries. Among the 1.5 million new cases estimated to occur annually, approximately 40% cases are actually reported (Cupolillo et al., 2000). Depending on the causative species, infection can manifest as cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), diffused cutaneous leishmaniasis (DCL) and visceral leishmaniasis (VL). *Leishmania amazonensis*, the species used in our study, has been found in many regions of Brazil and is associated with all four forms of this disease, which enhances its epidemiological significance (Barral et al., 1991; Leon et al., 1992).

L-Arginine plays a central role in the biosynthesis of various products, including nitric oxide (NO), urea, creatine phosphate, proline and polyamines via the ornithine decarboxylase (ODC) pathway. Several parasites are highly sensitive to NO and its derivatives. NO in micromolar concentrations is cytotoxic for microbial organisms and tumor cells. L-arginine is involved in the production of both NO, mediated by the different isofoms of nitric oxide synthase (NOS), and L-ornithine, mediated by arginase. Arginase hydrolyzes L-arginine to L-ornithine and urea and favors parasite growth. Polyamines have multiple roles in stabilizing nucleic acids and membranes, as well as regulating cell growth and differentiation (Moali et al., 1998; Vincendeau et al., 2003). The cellular production of NO absolutely depends on the availability of arginine because no other physiological amino acid or guanidine containing compound can substitute it as a substrate for NOS. NO synthesis must be accurately regulated, as NO is implicated in the pathophysiology of parasitic diseases (Vincendeau et al., 2003). Thus, NOS and arginase pathways have opposite biological effects (Hrabák et al., 1994). Previous results from our laboratory reported on the NO production by *Leishmania* sp. and the participation of the NO-*L. amazonensis* pathway in the host/parasite interaction (Géigel and Leon, 2003; Genestra et al., 2003a, b, c). Although macrophages trigger their defense mechanism to neutralize the parasite, there is evidence 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 (Temporal et al., 2005).The identification of parasite factors that induce arginase, as well as signaling involving NO, will make it possible to target the interference in the modulation NOS/arginase pathway (Vincendeau et al., 2003). Mesoionic compounds are a subclass of betaines, where the formal positive charge is associated with the ring atoms and the formal negative charge is associated either with ring atoms or an exocyclic atom. Mesoionic classes have provided numerous compounds with antibacterial, anti-inflammatory, antitrypanocidal and antitumor properties (da Silva et al., 2002; Senff-Ribeiro et al., 2004; da Silva Ferreira et al., 2008). Their potential value as biologically active substances is mainly due to the planar aromatic character and the separated regions with positive and negative charges. In this work, as a part of our research program on experimental leishmaniasis chemotherapy, we have tested six newly synthesized compounds (three thiadiazolium and three oxadiazolium derivatives) to determine their effect on the proliferation and on NOS/arginase pathways in *L. amazonensis* promastigotes and axenic amastigotes as well as their toxicity to mammalian cells.

2. Material and methods

2.1. Compounds

Mesoionic compounds 4-phenyl-5-(4′-X-phenyl)-1,3,4-thiadiazolium-2-phenylaminidechlorides (MI-H, MI-OCH_3 or MI-NO_2) and 3[N-4′-X-pheny]-1,2,3-oxadiazolium-5-olate (SID-H, SID-OCH_3 or SID-NO_2) were synthesized (Fig. 1) and their structure confirmed by ^{1}H-NMR, ^{13}C-NMR and mass spectrometry as previously reported (Moretto-dos-Reis, 2008; Dunkley and Thoman, 2003). Pentamidine isethionate (FilaxisLaboratory S.A.) was used as reference drug.

2.2. Reagents

Benzamidine, trypsin inhibitor, penicillin G, KCl, leupeptin, L-glutamine, streptomycin sulfate, Schneider's Insect Medium, MgCl2, phenylmethylsulfonyl fluoride (PMSF), N-1-naphtylethylenediamine, phosphoric acid, polyethyleneglycol, sulfanilamide, sucrose, TrisHCl, dithiotreitol (DTT), aprotinin, L-arginine, NADH, NADP, NADPH, L-NAME (N.-nitro-L-Arginine methyl esther), EDTA, ethanol, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide), DMSO, Triton X100, α-isonitrosopropienophenone, H_2SO_4, H_3PO_4, 5,6,7,8-tetrahydrobiopterin (H_4B), 2′,5′-ADP agarose, RPMI and DMSO were from Sigma Chemical Co., St. Louis, MO (USA).Glycerol, was from BioRad (USA). Fetal calf serum (FCS) was from Gibco BRL(USA).

2.3. Parasites

Infective promastigotes of *L. amazonensis* (MHOM/BR/77/LTB0016 strain), containing a high percentage (~73%) of metacyclic forms (evaluated through complement lysis assay were grown at 26 °C at pH 7.2 in Schneider's insect medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS). *L. amazonensis* axenic amastigotes were cultured at 32 °C in pH 5.5 in the same medium, supplemented with 20% (v/v) FCS, 0.5 mM HEPES and 0.5 mM glutamine. (Cysne-Finkelstein et al., 1998).

2.4. Drug assay

The in vitro efficacy of the mesoionic compounds was verified on promastigotes and axenic amastigotes of *L. amazonensis*. Parasites, after been harvested from the medium, were counted in Neubauer's chamber and adjusted to a concentration of 4×10^6 parasites/mL. Mesoionic compounds were added to the above parasite cultures for screening, using a 96-well microtitre plate, in a concentration range of 320–0.15 μg/mL solubilized in dimethyl sulphoxide (DMSO). Pentamidine isethionate was used as reference drug (Canto-Cavalheiro et al., 1997). After 24 h of incubation at specific temperatures (26 °C for promastigotes and 32 °C for axenic amastigotes), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) was added to the samples and the absorbance was read in a wavelength at 570 nm (MicroQuant