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Effects of trans-stilbene and terphenyl compounds on different strains of *Leishmania* and on cytokines production from infected macrophages

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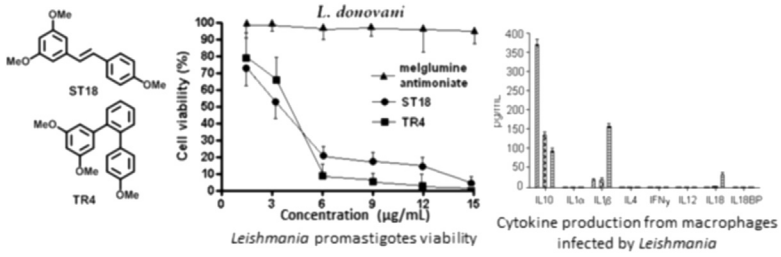
HIGHLIGHTS

- Stilbene ST18 and terphenyl TR4 were evaluated in 6 different species of *Leishmania*.
- ST18 and TR4 leishmanicidal activity was higher than that of Glucantime®.
- ST18 and the TR4 were also studied in *Leishmania* infected macrophages and the level of cytokines produced was determined.
- TR4 modulated cytokines level implicated in regulation of immunity against leishmaniasis.

GRAPHICAL ABSTRACT



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ABSTRACT

Most of the antileishmanial modern therapies are not satisfactory due to high toxicity or emergence of resistance and high cost of treatment. Previously, we observed that two compounds of a small library of trans-stilbene and terphenyl derivatives, ST18 and TR4, presented the best activity and safety profiles against *Leishmania infantum* promastigotes and amastigotes. In the present study we evaluated the effects of ST18 and the TR4 in 6 different species of *Leishmania* and the modifications induced by these two compounds in the production of 8 different cytokines from infected macrophages. We observed that TR4 was potently active in all *Leishmania* species tested in the study showing a leishmanicidal activity higher than that of ST18 and meglumine antimoniate in the most of the species. Moreover, TR4 was able to decrease the levels of IL-10, a cytokine able to render the host macrophage inactive allowing the persistence of parasites inside its phagolysosome, and increase the levels of IL-1β, a cytokine important for host resistance to *Leishmania* infection by inducible iNOS-mediated production of NO, and IL-18, a cytokine implicated in the development of Th1-type immune response.

1. Introduction

Leishmaniasis is a disease caused by an intracellular protozoan parasite (genus *Leishmania*) transmitted by the bite of a female phlebotomine sandfly (Kevric et al., 2015; Savoia, 2015). Leishmaniasis is prevalent in 98 countries with an incidence of 1.3

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million of new cases each year. The visceral form causes 300,000 cases (90% in Bangladesh, Brazil, Ethiopia, India, Nepal, South Sudan and Sudan) and one million belong to the cutaneous (mostly in Afghanistan, Algeria, Brazil, Colombia, Iran, Pakistan, Peru, Saudi Arabia, Syria and Tunisia) or mucocutaneous forms (especially in Brazil, Peru and Bolivia) (Alvar et al., 2012; Schönian et al., 2011).

There are about 53 species of *Leishmania* and 20 of them are known to be infective to humans. Pentavalent antimonials sodium stibogluconate and meglumine antimonate (Glucantime®) remain the first line treatment for all clinical forms of leishmaniasis, but there is a variable therapeutic response that could be correlated to the intrinsic difference in species sensitivity to the drug. Phenotypic diversity is observed not only between species, but also even in virulence levels among clones which, upon interaction with the host's immunological response, contribute to determine the observed clinical pleiotropy and affect the efficiency of therapy (Garin et al., 2001; Arevalo et al., 2007). Immunological response against *Leishmania* parasites is complex and has been associated with generation of T-helper (Th) 1 and Th2 responses respectively and activation of macrophages that produce inducible nitric oxide synthase (iNOS) and NO thus killing intracellular parasites and taking control of the disease (Murray and Nathan, 1999; Wei et al., 1995). However, during the phase of active disease patients generally exhibit a marked immunosuppression, and their peripheral blood mononuclear cells (PBMC) fail to respond when stimulated with leishmanial antigens *in vitro*. Initially this immunosuppression was thought to be associated with a Th2-type immune response seen as elevated levels of interleukin (IL) 4 and/or IL-13 (Sundar et al., 2002; Nylén et al., 2007). In contrast, other studies have shown in patients with active disease elevated levels of Th1 associated interleukins (IL-1, IL-6, IL-8, IL-12, IL-15), type 1 interferon (IFN) γ and tumor necrosis factor (TNF) α (Nylén et al., 2007; Ansari et al., 2006; Kurkjian et al., 2006) suggesting that other mechanisms contribute to the pathogenesis of immunosuppression. Recent studies indicate that IL-10 plays a main role in many of the immunologic defects associated with *Leishmania* infection. Patients with active leishmaniasis have elevated levels of IL-10 in serum as well as enhanced IL-10 mRNA levels in spleen, lymph nodes, and bone marrow (Ghalib et al., 1995). IL-10 inhibit the killing of amastigotes in macrophages by down-regulating the production of TNF-α and NO and IL-10 neutralization is able to promote parasite killing by increasing the levels of both TNF-α and IFN-γ (Nylén et al., 2007; Gautam et al., 2011). These findings strongly support a role for IL-10 in promoting pathology of leishmaniasis and suggest that targeting IL-10 may be a method to improve leishmaniasis therapy.

Several natural and synthetic stilbenoids have been studied for their leishmanicidal properties (Fuchino et al., 2013; Getti et al., 2006) and some of them, including resveratrol and piceatannol have shown antileishmanial activity *in vitro* (Ferreira et al., 2014; Duarte et al., 2008). Stilbene-based compounds are widely represented in nature and have become of particular interest to chemists and biologists because of their wide range of biological effects including those on human immune cell function (Woode et al., 2015; Soto et al., 2011; Falchetti et al., 2001). Recently, we evaluated the antileishmanial activity of a series of new trans-stilbene derivatives, in which a variety of substituents were introduced at positions 2′, 3′and 4′ of the stilbene scaffold while the 3,5-dimethoxy motif was maintained (Tolomeo et al., 2013; Castelli et al., 2016). Additionally as a further development of that project, we studied a series of terphenyl derivatives incorporating a phenyl ring as a bioisosteric substitution of the stilbene alkenyl bridge (Castelli et al., 2016). Two compounds of the series, namely trans-1,3-dimethoxy-5-(4-methoxystyryl)benzene (ST18) and 3,4″,5-trimethoxy-1,1′:2′,1″-terphenyl (TR4) (Fig. 1), presented the best activity and safety profiles. TR4 showed a leshmanicidal activity higher than pentostam and was able to induce apoptosis selectively in *Leishmania infantum* while saving macrophages and primary epithelial cells. Considering the intrinsic difference in species sensitivity toward antileishmanial drugs and the role of cytokines produced by macrophages in promoting pathology of leishmaniasis and drug resistance, in the present study we evaluated the effects of ST18 and the TR4 (Fig. 1) in 6 different species of *Leishmania*; moreover, we evaluated the modifications induced by these two compounds in the production of 8 different cytokines from infected macrophages. We observed that TR4 was more active than meglumine antimoniate in all *Leishmania* species tested in the study. Moreover, TR4 was able to decrease the levels of IL-10 and increase the levels of IL-1β and IL-18 produced *in vitro* by macrophages infected by *L. infantum.*

2. Material and methods

2.1. Parasites cultures

Leishmania infantum (*L. infantum* MHON/TN/80/IPT1 MON1) received from the Higher Institute of Health, Rome, Italy, and strains of *L. aethiopica* (MHOM/ES/72/L100), *L. braziliensis* (MHON/BR/75/M2904) *L. donovani* (MHOM/IN/80/DD8) *L. major* (MHOM/SU/73/5ASKH) *L. tropica* (MHOM/SU/74/K27) *L. amazonensis* (IFLA/Br/67/PH8) in promastigote forms, taken from strain collection of the Centro Nacional de Microbiología (National Microbiology Centre), Instituto de Salud Carlos III, Madrid, Spain, were cultured at 25 °C and pH 7.18 in RPMI-PY medium, which consisted of RPMI 1640 (Sigma R0883) supplemented with equal volume of Peptone-yeast medium, 10% fetal bovine serum (FBS, FBS, Gibco RPMI Media 1640, Invitrogen, Carlsbad, California), 1% glutamine (G6392, Sigma), 250 mg/mL gentamicin (G3632, Sigma) and 500 mg/mL of 5-fluorocytosine (F7129, Sigma) (Castelli et al., 2014).

2.2. Compounds

2.2.1. Synthesis

Trans-1,3-dimethoxy-5-(4-methoxystyryl)benzene (ST18) was synthesized as previously reported by Kim et al. (2002); 3,4″,5-trimethoxy-1,1′:2′,1″-terphenyl (TR4) was prepared as previously described by us (Roberti et al., 2006). The purity of compound was determined by elemental analyses and was ≥97%.

2.2.2. Sample preparation

Each compound was dissolved in dimethyl sulfoxide (DMSO) in a stock solution at a concentration of 20 mM, stored at −20 °C and protected from light. In each experiment DMSO never exceeded 0.2% and this percentage did not interfere with cell growth. Meglumine antimoniate was obtained from a commercial source (1.5 gr vials of Glucantime, Aventis Pharma, Milano, Italy) and dissolved initially in PBS (Sigma), and then RPMI-1640 medium to prepare serial dilutions.

2.3. Promastigotes viability assay

A number of 4×10^6/mL promastigotes of *Leishmania aethiopica*, *braziliensis*, *donovani*, *major*, *tropica*, *amazonensis* were suspended in 25 cm^2 flasks (Falcon) containing 10 mL of RPMI-PY medium and treated with serial concentrations (1, 3, 6, 9, 12, 15, 18, 21, 24 μg/mL) of each compound respectively comparing the data obtained with the same concentrations of meglumine antimoniate. After 48 h of treatment the parasites were centrifugated and resuspended in 1 mL of RPMI-PY medium. The suspension of *Leishmania* from each treatment was mixed with 0.4% trypan blue solution at a ratio of 3:1