

TTAS a new stilbene derivative that induces apoptosis in *Leishmania infantum*

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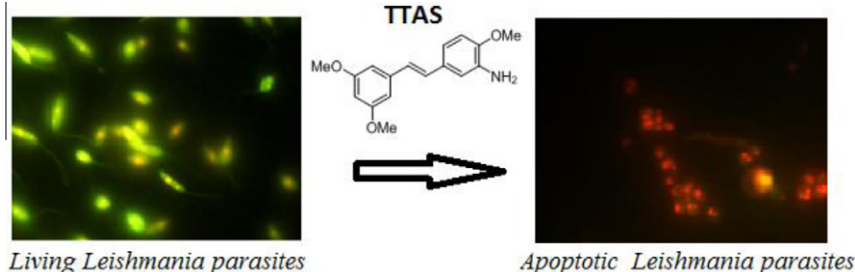
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HIGHLIGHTS

- ▶ We evaluated the Leishmanicidal activity of a pool of new stilbene derivatives.
- ▶ *Trans*-3,4',5-trimethoxy-3'-amino-stilbene (TTAS) is the most active stilbene.
- ▶ TTAS induces apoptosis in *Leishmania* parasites.
- ▶ *Leishmania* tubulin is the putative target of TTAS.

GRAPHICAL ABSTRACT



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ABSTRACT

Leishmania parasites are able to undergo apoptosis (programmed cell death), similarly to mammalian cells. Recently it was demonstrated in vitro the anti-leishmanial effect of some natural and synthetic stilbenoids including resveratrol and piceatannol. In this study we evaluated the Leishmanicidal activity of a pool of stilbene derivatives which had previously shown high apoptotic efficacy against neoplastic cells. All the compounds tested were capable to decrease the parasite viability in a dose-dependent manner. *Trans*-stilbenes proved to be markedly more effective than *cis*-isomers. This was different from that observed in tumor cells in which *cis*-stilbenes were more potent cytotoxic agents. *Trans*-3,4',5-trimethoxy-3'-amino-stilbene (TTAS) was the most active stilbene showing in *Leishmania infantum* a LD₅₀ value of 2.6 μg/mL. In contrast TTAS showed a low toxicity when tested on normal hemopoietic cells. This compound induced apoptosis in parasites by disrupting the mitochondrial membrane potential. Moreover it shows the ability to block *Leishmania* parasites in G₂-M phase of cell cycle in agreement with the data obtained by affinity chromatography that identify tubulin as the putative target of TTAS. In conclusion, our results indicate that some stilbene derivatives are highly effective as anti-leishmanial agents and TTAS represents a pro-apoptotic agent in *Leishmania* parasites that merit further in vivo investigation.

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1. Introduction

Leishmaniasis are globally widespread parasitic diseases caused by several protozoan parasites of the *Leishmania* genus. They are

transmitted to humans by the bite of an insect vector, generally the females sandfly of the genus *Phlebotomus* in the Old World, or *Lutzomyia* in the New World. Infections can vary from simple cutaneous leishmaniasis (CL) to mucocutaneous (MCL) and visceral leishmaniasis (VL) known as kala-azar, which is fatal if not treated. It is estimated that about half a million people die annually with VL and over 350 million people live at risk of this infection. Moreover, AIDS and other immunosuppressive conditions enhanced the risk of *Leishmania*-HIV co-infection and contribute to the appearance of new severe clinical forms of the disease. To date, the greatest

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prevalence of *Leishmania*-HIV co-infection has been in the Mediterranean basin (Cruz et al., 2006; Colomba et al., 2009). Current leishmaniasis treatment is based on chemotherapy, which relies on a handful of drugs with serious limitations such as high cost and toxicity, difficult route of administration, and lack of efficacy in endemic areas (Murray et al., 2005; Croft et al., 2006; Baiocco et al., 2009; Palumbo, 2009). Extensive evidence from studies in animal models indicates that good protection can be achieved by immunization, however, to date no vaccine is available and also for this reason there is an urgent need to develop safer, cheaper and more effective antileishmanial agents.

Natural products are potentially rich sources of novel active molecules that may serve as lead or structural template for drug discovery. In this context, in the last years several natural compounds and their synthetic analogs have been tested against *Leishmania*, and many of them have demonstrated potential as leishmanicidal agents (Changtam et al., 2010; Tipparaju et al., 2008; Marin et al., 2009; Mishra et al., 2009a,b).

Stilbene-based compounds are widely represented in nature (Shen et al., 2009), and have become of particular interest to chemists and biologists because of their wide range of biological effects including chemopreventive, antitumor, antioxidant, antimicrobial, anti-inflammatory and antihistaminic activities (Hart, 1981; Fremont, 2000; Young and Chaplin, 2004). Recently, several natural and synthetic stilbenoids have been studied for their leishmanicidal properties (del Rey et al., 1999; del Olmo et al., 2001; Getti et al., 2006), and some of them, including resveratrol (*trans*-3,4',5-trihydroxystilbene) (Kedzierski et al., 2007) and piceatannol (*trans*-3,3',4',5-tetrahydroxystilbene) (Duarte et al., 2008), have shown anti-leishmanial activity *in vitro*.

In previous works, we have described the synthesis of a small library of *cis*- and *trans*-resveratrol based derivatives with the aim of discovering new lead compounds with anticancer potential (Roberti et al., 2003). The best results were obtained with *cis*-3,4',5-trimethoxy-3'-hydroxy-stilbene **1** and its 3'-amino derivative **2**, which showed pro-apoptotic activity at nanomolar concentrations in HL60 leukemia cells. We have also demonstrated that pterostilbene **3** and 3'-hydroxy pterostilbene **4** possess interesting antileukemic properties and may constitute effective and powerful drugs in MDR and apoptosis-resistant hematological malignancies. Notably, the toxicity of these compounds, evaluated on normal hemopoietic cells (CFU-GM), was very low (Tolomeo et al., 2005; Cao et al., 2008).

In light of the above, here we present a study in which we evaluated the anti-leishmanial activity of stilbenes **1–2,4** together with their correspondent isomers **5–7** (**1** vs **5**, **2** vs **6**, **4** vs **7**, Table 1). Moreover we tested the activity of pterostilbene **3**.

Compound **6**, namely *trans*-3,4',5-trimethoxy-3'-amino-stilbene (TTAS), was the most active derivative showing a LD₅₀ value of 2.6 µg/mL. This finding prompted us to address the questions regarding the cellular target and the molecular mechanism underlying the observed toxicity toward the parasite. To this end we carried out a chemical proteomic analysis that led us to the identification of tubulin as a putative target of TTAS. Moreover, from the loss in mitochondrial membrane potential in *Leishmania* parasite, we hypothesize the activation of mitochondrial apoptotic pathway.

2. Materials and methods

2.1. Promastigote culture and cell treatments

The *Leishmania infantum* strain MON1/IPT1 promastigotes were cultured in blood agar as previously described (Sengupta et al., 1999). Briefly, the cells were routinely maintained on solid blood agar slants containing 1% glucose, 5.2% brain heart infusion agar extract and rabbit blood (6% v/v) with gentamycin at a final con-

centration of 1–1.5 mg mL⁻¹ of medium at 25 °C. Axenic amastigotes were obtained from promastigote cultures of *Leishmania infantum* strain MON1/IPT1. Promastigotes growing in log phase were harvested, washed in PBS, adjusted to a concentration of 10⁶ - parasites/mL in fresh medium at pH 5 and incubated at 32 °C. Complete transformation into amastigotes was achieved within 10 days of incubation. For experimental purposes, exponentially growing cells were collected and resuspended in RPMI medium supplemented with 10% fetal calf serum to achieve a culture density of 10⁶ cells/mL. Cells were dispensed in 24-well culture plates and appropriate concentrations of each compound were added.

2.2. Evaluation of live and dead parasites

To evaluate the leishmanicidal activity of stilbene-based compounds the percentage of live and dead parasites was determined morphologically after labeling with acridine orange (100 µg/mL) and ethidium bromide (100 µg/mL). After 48 h exposure to each compound, parasites (1 × 10⁶) were centrifuged and the pellet was resuspended in 25 µL of the dye mixture. Ten µL of the mixture was examined in oil immersion with a 100× objective using a fluorescence microscope Nikon Eclipse E200. Live parasites were determined by the uptake of acridine orange (green fluorescence) and exclusion of ethidium bromide (red fluorescence). Dead parasites was determined by the uptake of ethidium bromide (red fluorescence). The percentage of parasite viability was determined after counting at least 300 parasites.

2.3. Clonal assays

To evaluate the cytotoxic effects of stilbenes on normal hemopoietic progenitor cells, a clonal assay for CFU-GM (colony-forming units-granulocyte macrophage) was performed. Bone marrow mononucleated cells were obtained from bone marrow aspirates of five normal volunteers. Bone marrow (3–5 mL) was diluted in RPMI 1640, layered over a Ficoll-Hypaque gradient (density, 1.077), centrifuged at 400g for 30 min, and the interface mononuclear cells were collected. The interface cells were washed 3 times in PBS, counted, and resuspended at a concentration of 1 × 10⁵ in MEM containing 0.9% methylcellulose, 30% FCS, 10⁻⁴ M beta-mercaptoethanol, 5% medium conditioned by leukocytes in the presence of phytohemagglutinin (PHA-LCM) in 15 mm plastic dishes. After 7 days of culture at 37 °C in an environment of 5% CO₂ and 100% humidity, the number of CFU-GM, was evaluated.

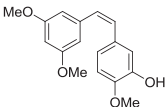
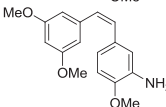
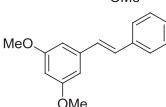
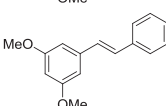
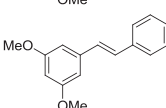
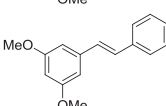
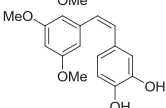
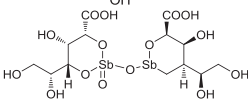
2.4. Flow cytometry analysis of cell cycle

Parasites were incubated for 24 h with the stilbene-based compounds at 26 °C. Afterward, the parasites were washed 3 times with PBS containing 0.02 M EDTA to avoid clumps and were then fixed with cold methanol for 24 h. The parasites were resuspended in 0.5 mL of PBS containing RNase I (50 µg/mL) and PI (25 µg/mL) and were then incubated at 25 °C for 20 min. The material was kept on ice until analysis. The stained parasites were analyzed in a single-parameter frequency histograms by using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

2.5. Determination of apoptosis by Annexin V

Externalization of phosphatidylserine on the outer membrane of parasites with and without treatment was determined by using Annexin V labeling kit following the manufacturer's protocol (Annexin-V-FITC Apoptosis Detection Kit Alexis, Lausen, Switzerland) (Singh et al., 2005). Briefly, parasites (2 × 10⁶) were washed with PBS and centrifuged at 500g for 5 min. The pellet was suspended in 100 µL of staining solution containing FITC-conjugated Annex-

Table 1
LD₅₀ values (μg/mL) of stilbenes 1–7 against the *Leishmania infantum* strain IPT1.

Compounds	Structure	Promastigotes LD ₅₀ (μg/mL)	Amastigotes LD ₅₀ (μg/mL)
1		17.5 ± 2.3	28.6 ± 3.4
2		18 ± 1.5	30.2 ± 4
3		7.6 ± 3	10.3 ± 1.8
4		6.5 ± 0.8	9.7 ± 2.1
5		10.9 ± 1.8	22.6 ± 3.9
6 (TTAS)		2.6 ± 0.4	4.3 ± 0.6
7		16.1 ± 3.2	28.4 ± 3.5
Pentostan		2.1 ± 0.5	8.3 ± 0.9

Values represent the mean ± SE for four independent experiments.

in-V and propidium iodide (Annexin-V-Fluos Staining Kit, Roche Molecular Biochemicals, Mannheim, Germany) and incubated for 15 min at 20 °C. Annexin V positive parasites were determined by flow cytometry (Becton Dickinson).

2.6. Measurement of mitochondrial membrane potential

Mitochondrial transmembrane potential was investigated using a JC-1 probe. JC-1 aggregates in mitochondria and fluoresces red at higher transmembrane potential but at lower transmembrane potentials it cannot accumulate in the mitochondria and remains as monomers in the cytoplasm that fluoresce green. Briefly, cells were labeled for 10 min with 10 μM JC-1 (BD™ MitoScreen JC-1 Kit, BD Biosciences Pharmingen, San Diego, CA) at 37 °C and washed 2 times. Multi-parameter flow cytometry analysis was performed by a Becton Dickinson flow cytometer.

2.7. Affinity chromatography and identification by mass spectroscopy

Compound **6** was immobilized via the primary amino group on epoxy-activated agarose providing a 12-atoms spacer (1,4-bis(2:3-epoxypropoxy)-butane). Swollen and thoroughly washed matrix was resuspended in two volumes of 25–100 mM ligand dissolved in 50% DMF/50 mM Na₂CO₃, 50 mM NaCl, pH 9.5. Coupling was performed for 16 h at 25 °C. After 5 washes with 50% DMF/50 mM Na₂CO₃, 50 mM NaCl, pH 9.5, remaining reactive groups were blocked with 1 M ethanolamine (pH 8) and thoroughly

washed with low/high pH buffers. A control matrix was prepared without ligand and treated as described above. Direct absorbance scans of the immobilized ligand on the matrix resuspended in 50% glycerol solution (v/v) clearly confirmed successful coupling (data not shown). The amount of compound bound to the matrix was determined by back calculation of amount of compound applied and amount recovered by UV determination. Routinely 50–80 μmol/mL compound were bound. The resin was incubated in 2.5 volumes of *Leishmania infantum* total cell lysate (12 mg/mL protein) at 4 °C for 3 h. After washing with lysis buffer (4 times), the matrix was heated at 95 °C for 5 min with Laemmli sample buffer, directly loaded and separated by 12% SDS-PAGE. Resolved proteins were visualized by silver staining. The control matrix was incubated with the same amount of lysate and treated equally. Protein bands were excise from the gels and fragments resulting from trypsin digestion were analyzed by LC/ESI/MS/MS-QTOF mass spectrometry. Data base searches were performed by using the ProteinLynx Global Server (all species) and Mascot (all species) search programs. A score >48 indicates identity ($p < 0.05$).

3. Results

3.1. Cytotoxicity

Compounds **1–7** were tested for leishmanicidal activity against promastigotes and amastigotes of *Leishmania infantum* (IPT1 strain). Parasites were incubated in presence of different concentrations

of each compound and viability was evaluated after 48 h, as described in materials and methods. The sample concentration causing 50% reduction in parasite viability (LD_{50}) was used as the parameter for leishmanicidal activity. LD_{50} values ranged from 18 $\mu\text{g}/\text{mL}$ to 2.6 $\mu\text{g}/\text{mL}$ in promastigotes and from 30.2 $\mu\text{g}/\text{mL}$ to 4.3 $\mu\text{g}/\text{mL}$ in amastigotes as depicted in Table 1. The *cis*-stilbene derivatives were less active than their corresponding *trans* isomers (compare **1** vs **5**, **2** vs **6**, **4** vs **7**). The pterostilbene **3** showed an activity comparable to that of its 3'-hydroxy derivative **4**. In the present study the most active compound was *trans*-3,4',5-trimeth-

oxy-3'-amino-stilbene (**6**, TTAS) that showed a LD_{50} of 2.6 $\mu\text{g}/\text{mL}$ (Table 1). Compared to Pentostan (sodium stibogluconate) used as reference compound, TTAS showed a slightly lower activity in promastigotes but was more active in amastigotes (Table 1).

Parasites exposed 48 h to TTAS and stained with the acridine orange and ethidium bromide mixture showed changes in morphology (like loss of cell volume and nuclear condensation) that shares many characteristics with metazoans apoptotic death (Fig. 1).

In order to evaluate the toxicity of TTAS on normal hemopoietic cells, bone marrow cells were treated with the compound and the number of CFU-GM was evaluated after 7 days of culture (Fig. 2). TTAS showed an IC_{50} on normal CFU-GM of 17.7 $\mu\text{g}/\text{mL}$, more than 6 times higher than that showed on the *Leishmania infantum* strain.

3.2. Cell cycle

The effect of TTAS on cell cycle distribution was analyzed in *Leishmania infantum*. Cells were cultured in the presence of 7 $\mu\text{g}/\text{mL}$ of TTAS for 24 h. Flow cytometric analysis of cell cycle was carried out after staining of parasites with propidium iodide. As shown in Fig. 3, TTAS induced the arrest of *Leishmanias* prevalently in G_2 -M phase of the cell cycle and an increase of the sub- G_1 apoptotic peak.

3.3. Determination of apoptosis by Annexin V

The Annexin V assay provides a simple and effective method to detect apoptosis. This assay takes advantage on the fact that (i) phosphatidylserine (PS) is translocated from the inner (cytoplasmic) leaflet of the plasma membrane to the outer (cell surface) leaflet soon after the induction of apoptosis, (ii) the Annexin V protein has a strong and specific affinity for PS.

As shown in Fig. 4, treatment of parasites with TTAS induces PS externalization (increase of cells in the bottom right quadrant of the flow cytometric dot plot) indicating that the cause of parasite death is the activation of apoptosis.

3.4. Mitochondrial membrane potential detection

TTAS induces change in mitochondrial membrane potential in *Leishmania infantum* strain IPT1. Studies in a variety of blood, hepatic, and immune cell models have shown that a fall in mitochondrial membrane potential is one of the earliest events in apoptosis (Susin et al., 1998). In fact, opening of the mitochondrial permeability transition pore has been demonstrated to induce depolarization of the transmembrane potential, release of apoptogenic factors and loss of oxidative phosphorylation. The loss of mitochondrial membrane potential has been observed to play a key role in drug-induced death in protozoans such as *Leishmania* (Sen et al., 2004a,b).

In this study, the modifications in mitochondrial membrane potential induced in *Leishmania infantum* strain IPT1, were evaluated using the fluorescent cationic dye JC-1. The JC-1 dye accumulates in the mitochondria of healthy cells as aggregates, which are fluorescent red in color. Upon the onset of apoptosis, the mitochondrial potential collapses and the JC-1 dye can no longer accumulate in the mitochondria and remains in the cytoplasm in a monomeric form which fluoresces green. The differential distribution of the red and green forms of the dye is easily analyzed by flow cytometry. The dye is highly specific and sensitive and gives very little background.

TTAS-induced loss in mitochondrial membrane potential in *Leishmania infantum* strain IPT1 is demonstrated by the decrease in red fluorescence (FL2) in the cytoplasm (Fig. 5).

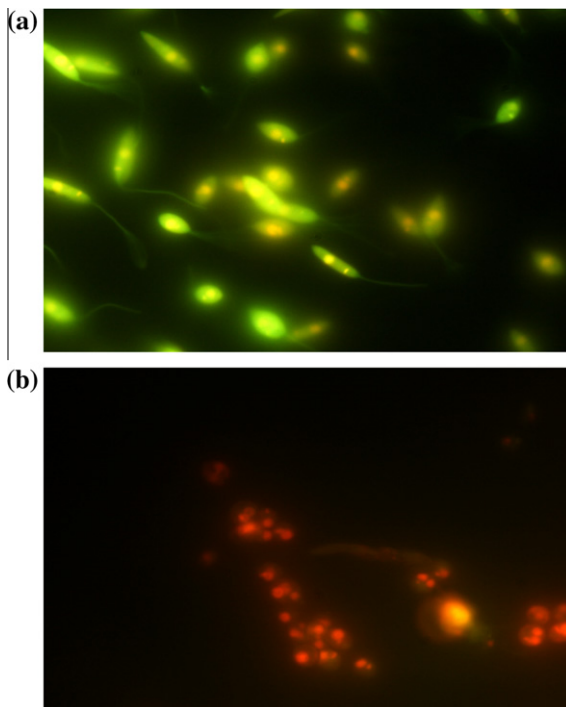


Fig. 1. Morphologic changes observed in *Leishmania infantum* after exposure to TTAS. Cells were exposed to a mixture of acridine orange and ethidium bromide as described in Section 2. Live parasites were determined by the uptake of acridine orange (green fluorescence) and exclusion of ethidium bromide (red fluorescence). Dead parasites were determined by the uptake of ethidium bromide (red fluorescence). (a) Control. (b) promastigotes exposed to 7 $\mu\text{g}/\text{mL}$ TTAS for 48 h; parasites uptake ethidium bromide showing a loss of volume and nuclear condensation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

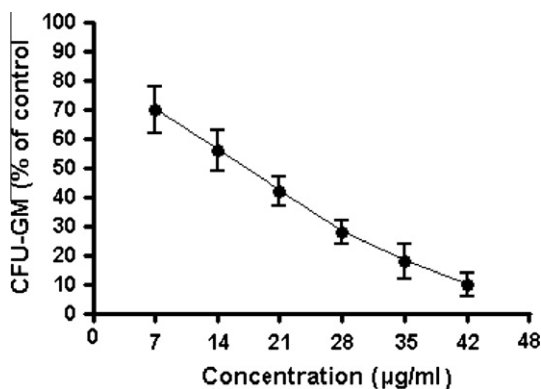


Fig. 2. Effects of compound TTAS on normal CFU-GM. Cells were exposed to different concentrations of TTAS. The number of CFU-GM was calculated after 7 days of cell culture.

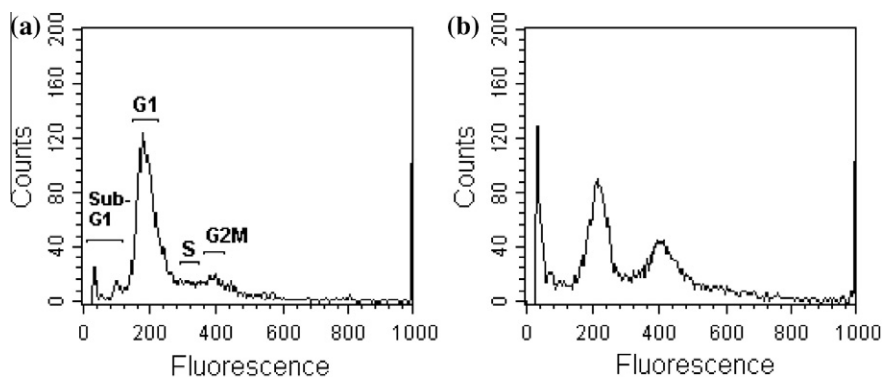


Fig. 3. Effects of compound TTAS on DNA content/parasite following treatment of *Leishmania* IPT1 strain for 24 h. The parasites were cultured without compound (control, panel a) or with TTAS used at 7 µg/mL (panel b). Cell cycle distribution was analyzed by the standard propidium iodide procedure. G₁, S, and G₂-M cells are indicated in panel a.

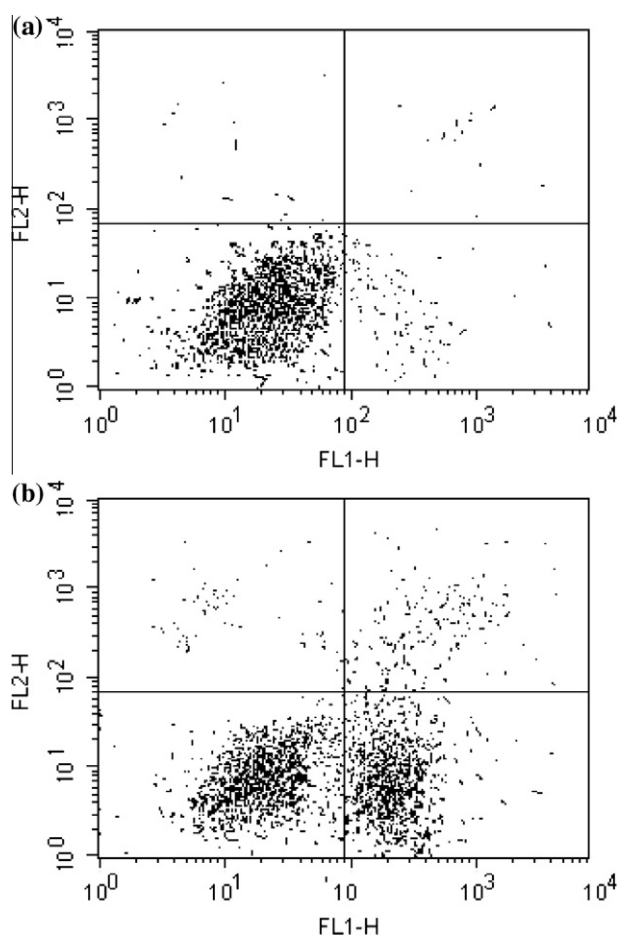


Fig. 4. Apoptosis evaluation by the Annexin-V test. Flow cytometric analysis of apoptotic *Leishmanias* after 48 h treatment without (panel a) or with (panel b) TTAS (7 µg/mL). Parasites were stained with Annexin V and propidium iodide. Cells in the bottom right quadrant of each dot plot represent the percentage of cells in early apoptosis (Annexin-V-positive and PI-negative). Data are representative of three separate experiments.

3.5. Immobilization of TTAS

In order to isolate and identify the intracellular target of TTAS in *Leishmania infantum* we decided to immobilize the compound and to isolate its target(s) by an affinity chromatography approach. The SAR based on the LD₅₀ values (Table 1) suggests that the addition of a hydrogen bond donor/acceptor in meta position (4 vs 3) of the phenyl moiety is not accompanied by a dramatic amelioration of

the activity. Thus, it has been hypothesized that the stilbene scaffold may represent the pharmacophore responsible for the activity. In line with this hypothesis, TTAS has been chosen for performing the affinity chromatography because it guarantees a unique binding to the matrix via the primary amino group which react more easily with the terminal epoxy group of the activated agarose than hydroxyl groups (as in compounds 5), providing an uncharged, hydrophilic and very stable 12-atoms spacer (Fig. 6). Direct absorbance scans of the immobilized ligand on the matrix resuspended in 50% glycerol solution (v/v) confirmed successful coupling (data not shown). Routinely 50–80 µmol/mL were bound.

3.6. β -tubulin binds to the immobilized compound TTAS

The total parasite lysate was prepared, loaded into the matrices and unbound material was removed as outlined in Section 2. The bound proteins were separated by SDS-PAGE and visualized by silver-staining (Fig. 7). The compound TTAS-derived matrix led to the detection of three distinct protein bands, two of which at higher molecular weights also being present in the control (Fig. 7). Trypsin digestion and peptide sequencing by LC/ESI/MS/MS-QTOF mass spectrometry identified the specific band at ~49 kDa as β -tubulin of *Leishmania infantum* (expected size of 49.7 kDa; score 82), whereas the other two corresponded to human keratin (scores 168 and 65). Human keratin that was present in both the control matrix and the TTAS-derived matrix was the predominant contamination which turned out to be present under any circumstances, and it was even found in new batches of matrix that were stored in sealed containers before first use.

4. Discussion

Leishmaniasis treatment is based on parenteral administration of highly toxic drugs including pentavalent antimonials, amphotericin B in its liposomal formulation (Ambisone) and pentamidine (Murray et al., 2005; Croft et al., 2006; Baiocco et al., 2009; Palumbo, 2009). Recently, oral administration of miltefosine has emerged as an alternative approach (Herwaldt, 1999). It was initially identified as an anticancer drug and its antiprotozoal activity was subsequently demonstrated against visceral and cutaneous leishmaniasis. Unfortunately, despite its great efficacy, miltefosine is limited by its extremely long half-life, and low therapeutic index.

In view of the foregoing facts, there is an urgent need for the development of antileishmania agents based on new molecular scaffold endowed with improved efficacy and lacking toxicity.

Recently, *trans*-resveratrol, a natural stilbene compound, has been studied for its leishmanicidal properties. Resveratrol has

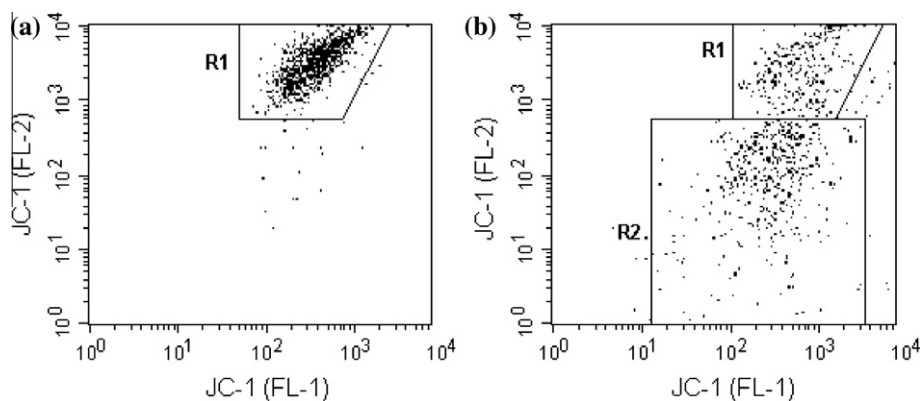


Fig. 5. Determination of TTAS-induced loss of mitochondrial potential in *Leishmania infantum* strain IPT1. Promastigotes treated with or without 7 µg/mL TTAS for 24 h were stained with potentiometric probe JC-1 and change in fluorescence were monitored by flow cytometry. Green color (FL1) indicates monomer at low concentrations and red color (FL2) indicates the aggregates at higher concentrations. JC-1 fluorescence is seen in both the FL-2 and FL-1 channels (R1) in untreated parasites (a). There is a significant increase in the number of parasites with lowered red fluorescence (R2) indicative of a change in the mitochondrial potential in parasites treated with TTAS (b). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

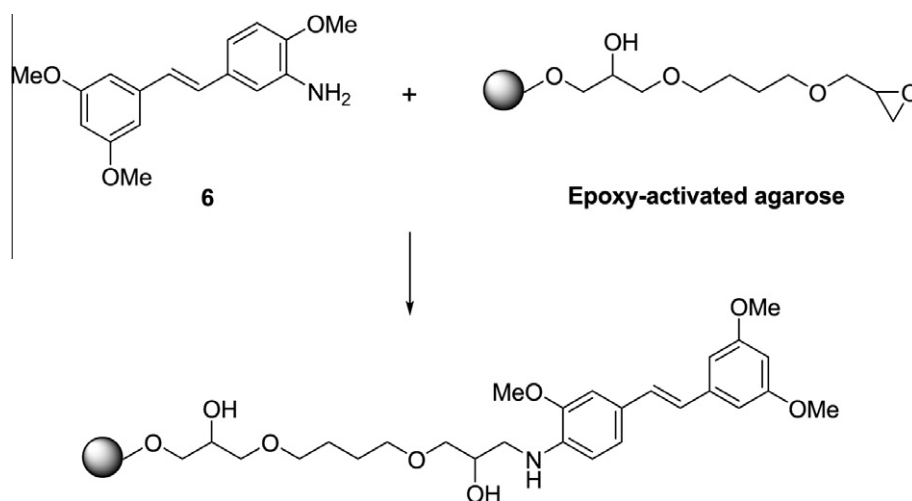


Fig. 6. Affinity matrix preparation. *Trans*-3,4',5'-trimethoxy-3'-amino-stilbene 6 (TTAS) was coupled to epoxy-activated agarose consisting of a 12-atoms spacer (1,4-bis(2:3-epoxypropoxy)butane) to form affinity matrix. The agarose beads are shown as spheres.

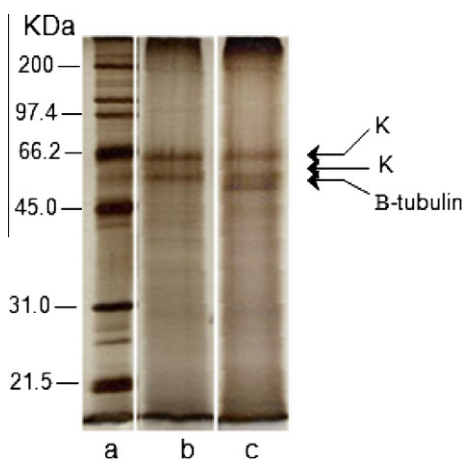


Fig. 7. SDS-PAGE (12%) analysis of proteins retained by the affinity matrix. Molecular mass marker (lane a). Samples of *Leishmania infantum* lysate (120 µg total protein content) were incubated with control beads (lane b), or affinity beads (lane c). Proteins retained by the matrices were directly separated by SDS-PAGE. Arrows indicate protein bands resulting from the compound derived matrices. K: human keratin.

shown antileishmanial activity when tested *in vitro* on *Leishmania major* promastigotes (Kedzierski et al., 2007). Microscopic examination indicated that the effect was leishmanicidal rather than leishmanistatic, as promastigote morphology indicated cells were dead, rather than their growth was slowed down. This is interesting considering that resveratrol is well-tolerated *in vivo* and it is not toxic at doses 1000 times higher than a normal daily intake (Juan et al., 2002). To obtain compounds endowed with higher antileishmanial activity, new resveratrol derivatives have been synthesized and tested on *Leishmania* parasites and normal human cells. However, most of them have shown a toxicity on normal fibroblasts and myeloid cells similar or higher than that observed in *Leishmania* parasites (Kedzierski et al., 2007).

Previously we have described the synthesis of new *cis*- and *trans*-resveratrol based derivatives with the aim of discovering new lead compounds with anticancer potential (Roberti et al., 2003; Tolomeo et al., 2005). In this work we investigated the anti-leishmania activity of some of them, with the aim of discovering new lead candidates or tools for the discovery of new therapeutically relevant targets.

Therefore stilbene derivatives 1–7 were assayed for their ability to inhibit the growth of *Leishmania infantum* promastigotes and amastigotes *in vitro*. All the compounds were capable to decrease

the parasite viability in a dose-dependent manner. As shown in Table 1, the *cis*-stilbene derivatives **1**, **2** and **7** showed an LD₅₀ higher than their correspondent *trans*-isomers **4**, **5** and **6** both in promastigotes and amastigotes. This was somewhat surprising to us because in our previous studies on leukemia cells, the *cis*-stilbene derivatives showed higher cytotoxic and pro-apoptotic activity than the parent *trans*-isomers (Roberti et al., 2003). The activity of pterostilbene **3** (LD₅₀ of 7.6 µg/mL) was comparable to that of its 3'-hydroxyl derivative **4** suggesting that the introduction of the second hydroxyl function in meta position is not influencing the leishmanicidal activity. Once again the result differed from that previously obtained on leukemia cells where the above modification resulted in about 40-fold increase in both cytotoxic and pro-apoptotic activity. In the present study the most active compound was *trans*-3,4',5'-trimethoxy-3'-amino-stilbene (**6**, TTAS) that showed a LD₅₀ of 2.6 µg/mL. Of interest, this compound showed an IC₅₀ on normal CFU-GM of 17.7 µg/mL, therefore more than 6 times higher than that showed on the *Leishmania infantum* strain. For this selectivity TTAS may be considered an interesting compound for the antileishmanial therapy development, therefore we tried to understand its antileishmanial mechanism of action. *Leishmania* parasites treated 24 h with TTAS were blocked prevalently in G₂-M phase of cell cycle and after 48 h of TTAS exposure *Leishmania* parasites were induced in apoptosis as shown by morphologic examination and Annexin V positivity. The loss of mitochondrial membrane potential suggests that the TTAS-induced apoptosis is mediated by the mitochondrial apoptotic pathway. To discover the main molecular target/s of TTAS in *Leishmania* parasites we took advantage of a chemical proteomic approach that was recently successfully applied by some of us (Kuettel et al., 2009). Thus the amino-stilbene derivative TTAS, chosen based on the presented activity data, was immobilized and the corresponding affinity matrix was used to find out potential targets. Indeed this approach identified β-tubulin as putative specific target of TTAS in *Leishmania* consistently with the ability of TTAS to block *Leishmania* parasites in G₂-M phase of cell cycle.

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