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In vitro antileishmanial activity of *trans*-stilbene and terphenyl compounds

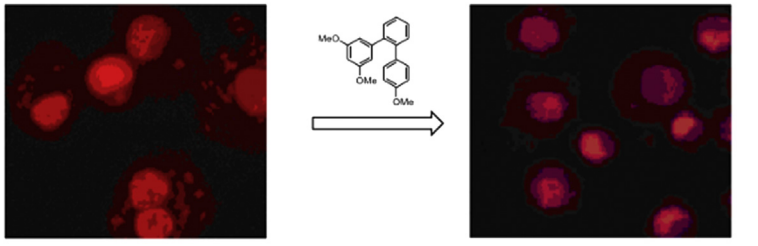
Germano Castelli [a], Federica Bruno [a], Fabrizio Vitale [a], Marinella Roberti [b], Claudia Colomba [d], Elisa Giacomini [c], Laura Guidotti [b], Antonio Cascio [d], Manlio Tolomeo [d, *]

[a] National Reference Center for Leishmaniasis (C.Re.Na.L.), Istituto Zooprofilattico Sperimentale della Sicilia, Via Gino Marinuzzi 3, 90129, Palermo, Italy
[b] Department of Pharmacy and Biotechnology, University of Bologna, Via Belmeloro 6, 40126, Bologna, Italy
[c] D3 Compunet, Istituto Italiano di Tecnologia, Via Morego 30, I-16163, Genova, Italy
[d] Department of Health Promotion Sciences, Section of Infectious Diseases, University of Palermo, Via del Vespro 129, 90127, Palermo, Italy

HIGHLIGHTS

- We evaluated the leishmanicidal activity of a pool of new stilbene and terphenyl derivatives.
- Therphenyl **11** showed a leshmanicidal activity higher than pentostam and TTAS.
- Therphenyl **11** induces apoptosis in *Leishmania* parasites while saving macrophages and normal cells.

GRAPHICAL ABSTRACT



Infected macrophages before treatment *Infected macrophages after treatment*

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ABSTRACT

Leishmaniasis are globally widespread parasitic diseases which often leads to death if left untreated. Currently available drugs present different drawbacks, so there is an urgent need to develop new, safe and cost-effective drugs against leishmaniasis. In this study we tested a small library of *trans*-stilbene and terphenyl derivatives against promastigote, amastigotes and intramacrophage amastigote forms of *Leishmania infantum*. Two compounds of the series, the *trans*-stilbene **3** and the terphenyl **11**, presented the best activity and safety profiles. Terphenyl **11** showed a leshmanicidal activity higher than pentostam and the ability to induce apoptosis selectively in *Leishmania infantum* while saving macrophages and primary epithelial cells. Our data indicate that terphenyl compounds, as well as stilbenes, are endowed with leishmanicidal activity, showing potential for further studies in the context of leishmanial therapy.

1. Introduction

Leishmaniasis is a disease caused by an intracellular protozoan parasite (Leishmania) transmitted by the bite of a sandfly. The clinical spectrum of leishmaniasis ranges from a self-resolving cutaneous ulcer to a mutilating mucocutaneous disease and even to a lethal systemic illness. Most of the antileishmanial modern

* Corresponding author. Department of Health Promotion Sciences, Section of Infectious Diseases, University of Palermo, Interdepartmental Research Centre on Clinical Oncology (C.I.R.O.C.), University of Palermo, Via del Vespro 129, 90127, Palermo, Italy.
E-mail address: mtolomeo@hotmail.com (M. Tolomeo).

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therapies are not satisfactory due to high toxicity or emergence of resistance and high cost of treatment (Monzote, 2009). The pentavalent antimonial drugs are used as the first line of treatment. The two available preparations, sodium stibogluconate (pentostam), produced in Great Britain, and meglumine antimonate (glucantime), produced in France, have similar efficacy. However, these drugs can induce important side effects such as cardiotoxicity, reversible renal failure, pancreatitis, anemia, leukopenia, rash, headache, abdominal pain, nausea, vomiting, arthralgia, myalgia, thrombocytopenia, and transaminase elevation. Moreover in the past 15 years the resistance against these drugs have become a clinical threat (Croft et al., 2006). Pentamidine, a pentavalent antimonial drug used as standard first-line treatment for leishmaniasis, has been reported to cause ventricular arrhythmias and nephrotoxicity. Recently, oral administration of miltefosine has emerged as an alternative approach (Herwaldt, 1999). Unfortunately, despite its great efficacy, miltefosine is limited by its extremely long half-life, low therapeutic index and development of resistance (Sundar and Murray, 2005). Liposomal amphotericin B has been used with increasing frequency to treat visceral leishmaniasis. It is the treatment of choice for immunocompetent patients in the Mediterranean region and the preferred drug for HIV/visceral leishmaniasis co-infection. However with the increasing use of amphotericin B in lipid formulation having longer half-lives, amphotericin B resistant mycoses are emerging (Croft et al., 2006). Therefore, the possibility of amphotericin B resistance in leishmaniasis cannot be ignored (Purkait et al., 2012; Brotherton et al., 2014). So there is an urgent need to develop new, safe and cost-effective drugs against leishmaniasis.

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 analogues 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; Hussain et al., 2014; Singh et al., 2014). Stilbenes-based compounds are widely represented in nature (Shen et al., 2009; Riviere et al., 2012) 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; Fuchino et al., 2013; de Almeida Machado et al., 2015) and some of them, including resveratrol (*trans*-3,4′,5-trihydroxystilbene, Fig. 1) (Kedzierski et al., 2007; Lucas and Kolodziej, 2013; Ferreiraa et al., 2014) and piceatannol (*trans*-3,3′,4′,5-tetrahydroxystilbene, Fig. 1) (Duarte et al., 2008), have shown anti-leishmania activity *in vitro*.

In light of the above, recently we described a study in which we evaluated the anti-leishmanial activity of *cis*- and *trans*-stilbenes (Tolomeo et al., 2013). All the tested *c*is-analogues were less potent than their corresponding *trans* isomer. Compound **1**, namely *trans*-3,4′,5-trimethoxy-3′-amino-stilbene (**TTAS,** Fig. 1), was the most active in *Leishmania infantum* (IC_{50} value of 2.6 μg/mL), while displaying a low toxicity on normal hemopoietic cells.

To further explore the structure-activity relationships (SAR) of this class of compounds we describe herein a study in which we evaluated the antileishmanial activity of a second series of *trans*-stilbenes 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 (**2**–**5**, Fig. 1). Additionally as a further development of this project, we study a series of terphenyl derivatives (**6**–**12**, Fig. 1) incorporating a phenyl ring as a bioisosteric substitution of the stilbene alkenyl bridge. Terphenyls **6**–**12** present the resveratrol-like pattern of oxygenated phenyl rings, *i.e*., one *para*-substituted and one di-*meta*-substituted phenyl connected by an unsaturated/aromatic system.

All the compounds evaluated herein were previously synthesized by us with the aim of discovering new lead with anticancer potential (Roberti et al., 2003, 2006; Pizzirani et al., 2008), except **4** that was reported by Kim et al. (2002). The compounds with the higher antileishmanial activity and a safety profile, *i.e*., no cytotoxic were further used to characterize their biological properties.

2. Material and method

2.1. Parasites cultures

A strain of *Leishmania infantum* promastigotes zimodeme 1 (MHON/TN/80/IPT1 MON1) received from the Higher Institute of Health of Rome (Italy) was cultured at 25 °C and pH 7.18 in RPMI-PY medium, which consisted of RPMI 1640 (Sigma R0883) supplemented with equal volume of Pepton-yeast medium, 10% fetal bovine serum (FBS), 1% glutamine, 250 μg/mL gentamicin and 500 μg/mL of 5-fluorocytosine (Castelli et al., 2014). To obtain amastigotes cells, promastigotes were cultivated in RPMI-PY, in which the pH was acidified with 1 N HCl to 5.4, and incubated at 37 °C.

2.2. Promastigotes and amastigotes viability assay

Promastigotes or amastigotes of *Leishmania infantum* (4×10^6/mL) were suspended in 25 cm^2 flasks (Falcon) containing 10 ml of RPMI-PY medium and treated with serial concentrations of each compound. 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 (vol/vol) (Dutta et al., 2005). The percentage of vitality of *Leishmania* was observed by counting in a Bürker hemocytometer for enumeration of stained and unstained cells, taken respectively as dead and living cells, in comparison with the control culture (100% viability).

2.3. Mammalian cell cytotoxicity

Potential cytotoxic action of each compound was checked by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT) assay on U937 (human Caucasian histiocytic lymphoma, ECACC 85011440, UK) and in primary epithelial cells of Cercopiteco (CPE). U937 and CPE cells were cultured in RPMI 1640 (Sigma R0883) supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin (100 IU/mL) and streptomycin (100 mg/mL). Cells were grown at 37 °C in 5% CO_2 and passaged twice a week. In each experiment, cells (10^5/well) were incubated into 96-well plates overnight in a humidified 5% CO_2 atmosphere at 37 °C to ensure cell adherence. After 24 h, cells were treated with increasing concentrations of each compound. Non-treated cells were included as a negative control. After 48 h incubation with each compound, the MTT (5 mg/mL) was added to each well and incubated at 37 °C for 4 h. Then the medium and MTT were removed, cells washed by PBS and 200 μL of DMSO were added to dissolve the formazan crystals. The absorbance was measured using a microplate reader Spectrostar Nano (BMG LabTech) at 570 nm. The reduction of MTT to insoluble formazan was done by the mitochondrial enzymes of viable cells and so is an indicator of cell viability. Therefore, decreases of absorbance indicate toxicity to the cell. The viability was calculated using the following formula: [(L2/L1) × 100], where L1 is the absorbance of control cells and L2 is the absorbance of treated cells. The 50%

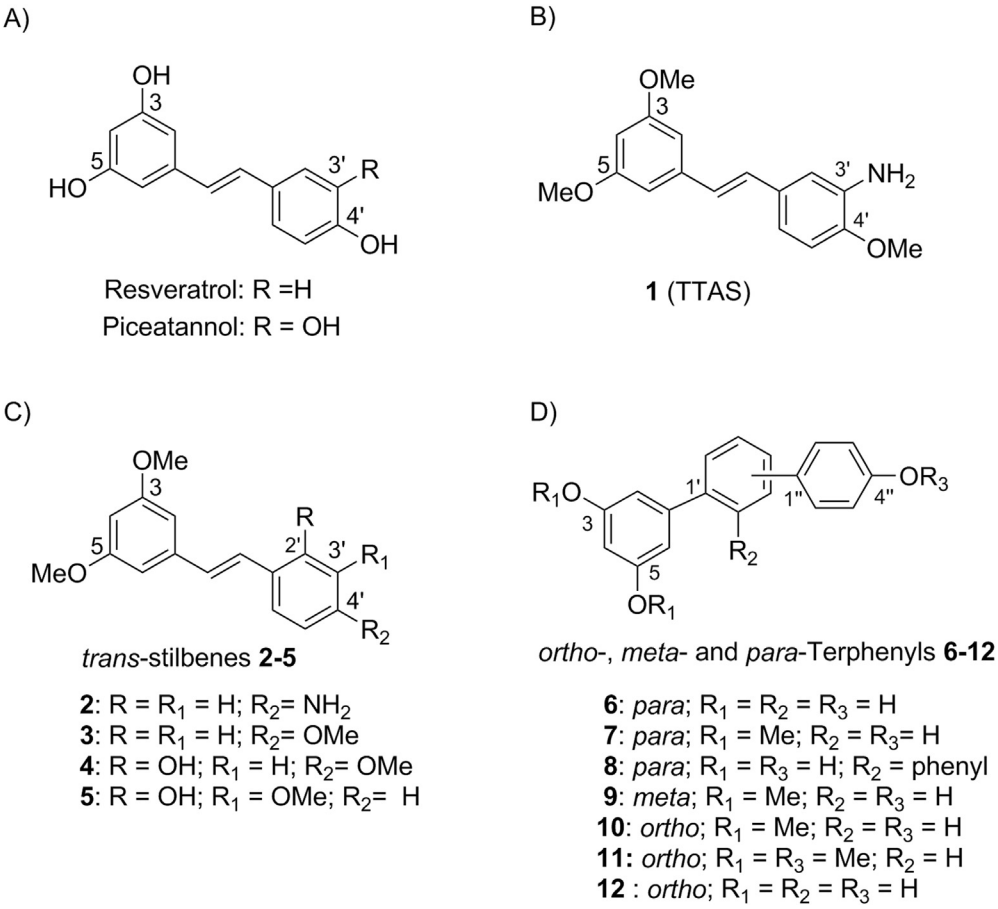


Fig. 1. Structures of stilbene and terphenyl compounds: A) Resveratrol and piceatannol; B) trans-3,4′,5-trimethoxy-3′-amino-stilbene **1** (TTAS); C) trans-stilbenes **2**–**5**; D) terphenyls **6**–**12**.

cytotoxic concentration (CC50), *i.e.*, the drug concentration that decreases the rate of cell vitality by 50%, was calculated by regression analysis (GraphPad software).

2.4. Effects of compounds in intracellular amastigotes

In order to evaluate the effects of compounds in cultures of macrophages infected with *Leishmania infantum* a macrophage infection protocol similar to that described by Sharma (Sharma et al., 2011) was used with some modifications. U937 monocytic cells (1×10^5 cells/mL) in the logarithmic phase of growth were plated onto chamber Lab Tek culture slides in 2.5 mL of RPMI 1640 (Sigma), 10% FBS medium containing 25 ng/mL of phorbol 12-myristate 13-acetate (PMA-Sigma) for 18 h to induce differentiation (Maia et al., 2007). After incubation, the medium was removed by washing twice with RPMI-1640 medium. Non adherent cells were removed, and the macrophages were further incubated overnight in RPMI 1640 medium supplemented with 10% FBS. Then adherent macrophages were infected with *Leishmania infantum* promastigotes at a parasite/macrophage ratio of 5:1 for 24 h at 37 °C in 5% CO_2. Free promastigotes were removed by three extensive washing with RPMI-1640 medium, and infected macrophages were either incubated 48 h in media alone (infection control) or with serial concentrations of each compound. With the aim of stain intracellular amastigotes, cells were fixed with iced methanol to permeabilize cell membrane to ethidium bromide and stained with 100 μg/mL ethidium bromide (De Souza Leao et al., 1995; Azzouz et al., 2005). The number of infected cells was determined by examining three coverslips for each treatment. At least 200 macrophages were counted by visual examination under 40× magnifications by using a fluorescence microscope Nikon Eclipse E200 (Nikon Instruments Europe, Amsterdam, Netherlands) to determine the number of resident amastigotes. The number of resident amastigotes was expressed as percentage of the control.

2.5. Morphological analysis of cell death

To evaluate the type of parasite death (necrosis or apoptosis) cells were stained with a mixture of acridine orange (100 μg/mL) and ethidium bromide (100 μg/mL) (Liegler et al., 1995; Ribble et al., 2005; Tolomeo et al., 2013). Promastigotes of *Leishmania infantum* (4×10^6/ml) were suspended in 50 cm^2 flasks (Falcon) containing 10 ml of RPMI-PY medium and treated with each compound (10 μg/mL). After 48 h of treatment promastigotes (1×10^6) were centrifuged and the pellet was resuspended in 25 μL of stained mixture and examined in oil immersion with a 100x objective using a fluorescence microscope (Nikon Eclipse E200). The characteristic nuclear phenotype exhibited by apoptotic cells (condensation of genomic DNA) was used to distinguish between normal and apoptotic cells. Viable or necrotic cells were identified by nuclei with green or red fluorescence, respectively. Apoptotic cells were detected by their condensed nuclei. In early apoptosis, only acridine orange will reach the nuclear material (green fluorescence), whereas, in the later phase, ethidium bromide will penetrate the cells as well (red fluorescence). Drug induced apoptosis and necrosis was also determined morphologically in macrophages

(differentiated U937 cells) and in primary epithelial cells of Cercopiteco (CPE) after labeling with acridine orange and ethidium bromide. Cells (2×10^5) were centrifuged and the pellet was resuspended in 25 μL of the dye mixture. Ten microliters of the mixture was examined in oil immersion with a 100x objective using a fluorescence microscope (Nikon Eclipse E200). Live cells were determined by the uptake of acridine orange (green fluorescence) and exclusion of ethidium bromide (red fluorescence) stain. Dead apoptotic and necrotic cells were identified by perinuclear condensation of chromatin stained by acridine orange or ethidium bromide, respectively, and by the formation of apoptotic bodies. The percentage of apoptotic cells was determined after counting at least 300 cells.

2.6. Cell cycle analysis by flow cytometry

Promastigotes (4×10^6) were incubated for 24 h with each compounds (8 μg/mL) at 26 °C. We use 8 μg/mL because the treatment of promastigotes with 10 μg/mL caused a too large recruitment of parasites in sub-G1 (apoptotic cells). Afterward, the *Leishmania infantum* 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 single-parameter frequency histograms by using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

2.7. 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, promastigotes (2×10^6) were washed with PBS and centrifuged at 500 g for 5 min. The pellet was suspended in 100 μL of staining solution containing FITC-conjugated Annex-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).

2.8. Statistical analysis

All susceptibility assays on *Leishmania infantum* promastigote and amastigote cells were performed by two observers in three replicates samples and repeated with three new batches of parasites. The mean and standard error of at least three experiments were determined. The differences between the mean values obtained for experimental groups were evaluated by the Student's t test. P-values of 0.05 or less were considered significant.

3. Results

3.1. Antileishmanial activity

Four *trans*-stilbenes derivatives (**2**–**5**, Table 1) and seven terphenyl derivatives (**6**–**12**, Table 1) were tested *in vitro* in a strain of *Leishmania infantum* promastigotes zimodeme 1 (MHON/TN/80/IPT1 MON1) (Fig. 1). The activity of these compounds was compared to that of pentostam, a pentavalent antimonial drug used as the first line of treatment of *Leishmania* infection and **TTAS**, a *trans*-stilbene derivative previously studied by us and endowed with potent anti-leishmanial activity (Tolomeo et al., 2013). The

Table 1
IC_{50} values (μg/ml) of *trans*-stilbene derivatives **1**–**5** and terphenyl derivatives **6**–**12** against promastigotes of *Leishmania infantum*.

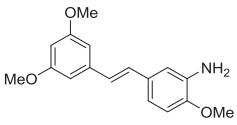
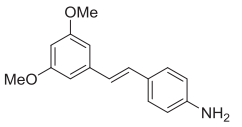
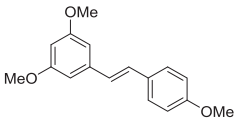
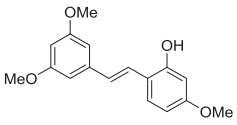
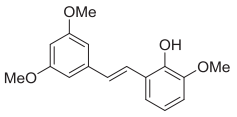
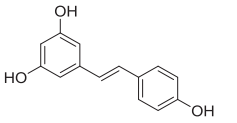
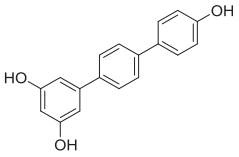
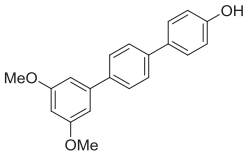
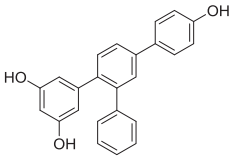
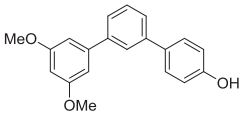
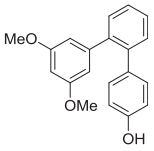
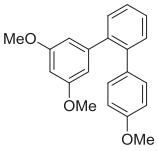
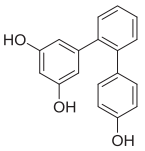
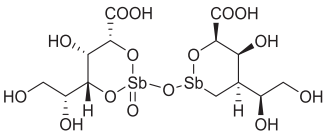
Compound	Structure	IC_{50} μg/mL
1 (TTAS)		2.6 ± 0.4
2		10.7 ± 0.8
3		2.1 ± 0.3
4		11 ± 0.2
5		4.2 ± 0.6
Resveratrol		>50
6		>50
7		5.3 ± 0.8
8		>50
9		3.8 ± 0.6
10		27 ± 4.1

Table 1 (*continued*)

Compound	Structure	IC_{50} μg/mL
11		1.4 ± 0.2
12		>50
Pentostam		2.1 ± 0.5

Values represent the mean ± SE for four independent experiments.

compound concentration causing 50% reduction in parasite viability (IC_{50}) was used as the parameter for leishmanicidal activity. As shown in Table 1 all *trans*-stilbene derivatives were endowed with leishmanicidal activity with a IC_{50} ranging from 2.1 μg/mL to 11 μg/mL. The most active *trans*-stilbene of this series was compound **3** that showed a leishmanicidal activity similar to pentostam and **TTAS**. The range of activity of terphenyl compounds (**6**–**12**) was very wide and the IC_{50} ranged from 1.4 μg/mL to more than 50 μg/mL (Table 1). Of interest, terphenyl **11** was slightly more active than pentostan and **TTAS**. Based on leishmanicidal activity data, compounds **3** and **11** were selected for further studies. In Fig. 2a and 2b are reported the dose-response curves of **3** and **11** in promastigotes and amastigotes respectively. Terphenyl **11** was slightly more active than *trans*-stilbene **3** either in promastigotes or in amastigotes. As shown in Fig. 2 and Table 2 both the compounds were more active in amastigotes than in promastigotes.

3.2. Effects in intracellular amastigotes

Upon entering the mammalian host, *Leishmania* parasites transform into the amastigote stage that reside inside the phagolysosomal vacuoles of macrophages. Therefore, it is important to evaluate the antileishmanial activity on intracellular amastigotes of any new potential compound. We evaluated the anti-amastigote

Table 2
IC_{50} (μg/mL) of **3** and **11** against promastigote, amastigote and intracellular amastigote forms of *Leishmania infantum*.

Leishmania infantum cells	Compounds	
	3	**11**
Promastigotes	2 ± 0.3	1.4 ± 0.2
Amastigotes	0.81 ± 0.09	0.65 ± 0.08
Intracellular Amastigotes	4.2 ± 0.7	3 ± 0.5

Values represent the mean ± SE for four independent experiments.

efficacy in U937 macrophage cells infected with *Leishmania infantum* as reported in material and methods. A dose-dependent decrease in the number of intra-macrophagic amastigotes was observed both with compounds **3** and **11** (Figs. 3 and 4). The IC_{50} was 4.2 μg/mL and 3 μg/mL for **3** and **11** respectively (Table 2).

3.3. Cytotoxic effects

In order to evaluate the toxicity of **3** and **11**, primary epithelial cells of Cercopiteco (CPE) and uninfected U937 macrophages were treated with these compounds and cytotoxicity was evaluated after

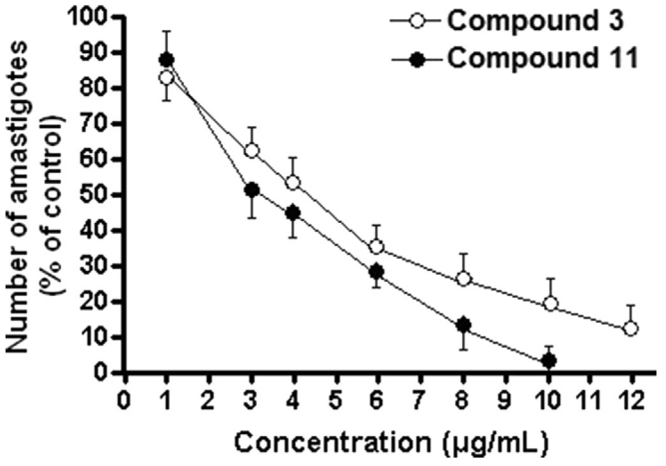


Fig. 3. Effects of compounds **3** and **11** in intracellular amastigotes. The number of intracellular amastigotes in the samples treated with **3** and **11** was expressed as percentage of the number of intracellular amastigotes in the control. At least 200 macrophages were counted by visual examination under 400× magnifications by using a fluorescence microscopy to determine the number of resident amastigotes. Bars indicate the mean ± SE from four independent experiments.

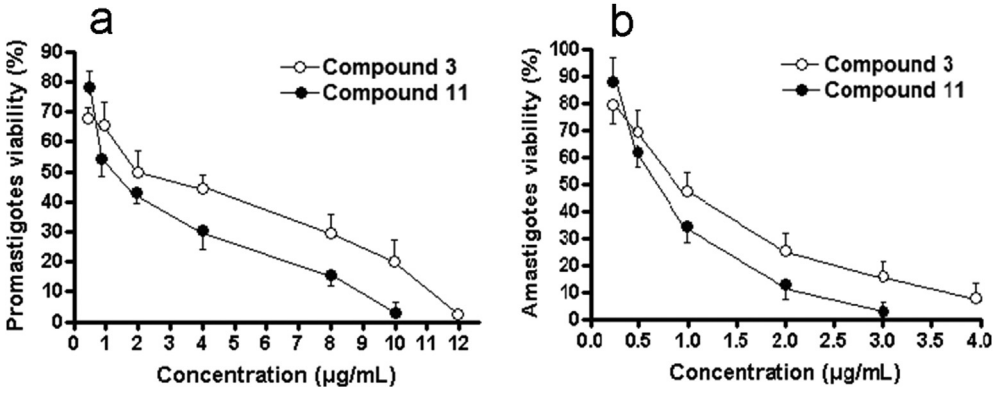


Fig. 2. Promastigotes (a) and amastigotes (b) viability after 48 h exposure to increasing concentrations of compounds **3** and **11**. Bars indicate the mean ± SE from four independent experiments.

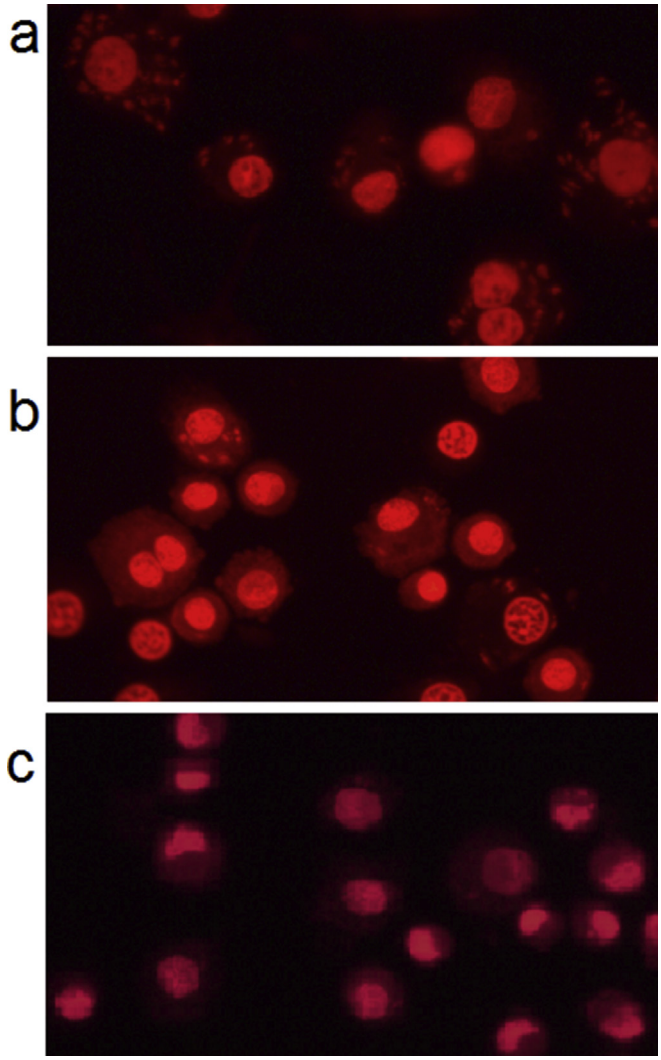


Fig. 4. Morphology of untreated infected macrophages (a), infected macrophages exposed to 10 μg/ml compound **3** for 48 h (b), and infected macrophages exposed to 10 μg/ml compound **11** for 48 h (c). Cells were fixed with iced methanol to permeabilize the cell membrane to ethidium bromide, stained with ethidium bromide and examined by using a fluorescence microscopy. Data are representative of three separate experiments.

48 h through MTT assay. For concentrations ranging from 1 μg/mL to 12 μg/mL **3** and **11** did not caused any reduction in CPE viability (Fig. 5a). Used at 12 μg/mL compound **3** caused on U937 macrophages a reduction of living cells of about 36% while compound **11** did not cause any cytotoxic effect (Fig. 5b).

3.4. Morphological alterations

Morphological alterations induced in parasites by the treatment with compound **3** and **11** were evaluated using a fluorescence microscope after labeling with acridine orange and ethidium bromide. The untreated parasites showed green fluorescence and classical morphology being slender, elongated and flagellated (Fig. 6a). Promastigotes treated with compound **3** or **11** revealed morphological changes that share many characteristics with apoptotic death of the metazoans (cell shrinkage, cytoplasmic and nuclear condensation) (Fig. 6b and 6c). The percentage of cells showing morphological alterations was correlated with the concentration of each drug (data not shown). After 48 h exposure to 10 μg/mL **3** or **11** the percentage of promastigotes with morphological alterations was 80% and 98% respectively; in contrast, macrophages exposed 48 h to 10 μg/mL **3** or **11** do not show any morphological change compared to the control (data not shown). Therefore, compounds **3** and **11** were able to induce morphological aspects of apoptosis only in parasites and not in macrophages. To confirm these data the Annexin V assay was carried out on *Leishmania infantum* promastigotes and macrophages.

3.5. Apoptosis in leishmania promastigotes cells

The Annexin V assay provides a simple and effective method to detect apoptosis or necrosis. 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, and (ii) the Annexin V protein has a strong and specific affinity for PS. Staining with Annexin V was used in conjunction with propidium iodide (PI), for identification of early apoptotic, late apoptotic and necrotic cells. Flow cytometric detection of Annexin V positive cells identifies the percentage of cells that are in early (Annexin V positive cells in lower right quadrant) and late apoptosis (Annexin V and PI positive cells double stained in the upper right quadrant); Annexin V negative/PI positive cells identifies the percentage of necrotic cells. As shown in Fig. 7, treatment of parasites with 10 μg/mL compound **3** (Fig. 7b) and compound **11** (Fig. 7c) for 48 h induced PS externalization (increase of cells in the right quadrants of the flow cytometric dot plot) indicating that the cause of parasite death is the activation of apoptosis. In contrast, these compounds did not induce any PS externalization in macrophages (Fig. 7 d–f).

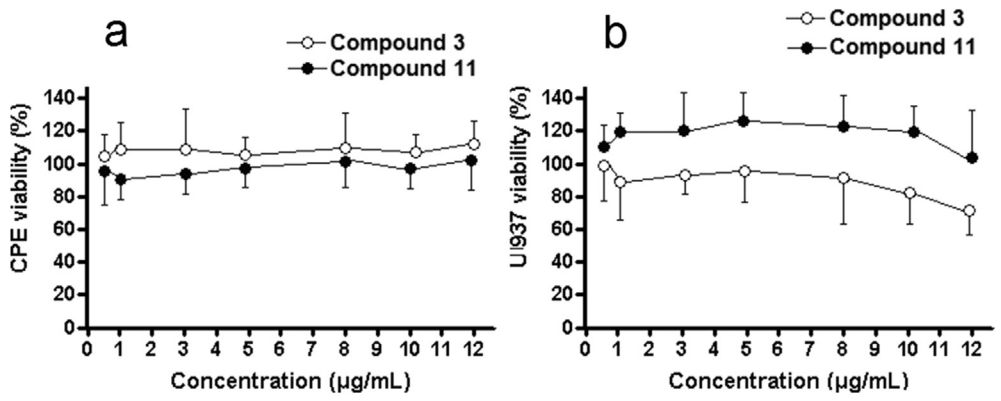


Fig. 5. Cytotoxic effects of compounds **3** and **11** in CPE cells and U937 macrophages. Bars indicate the mean ± SE from four independent experiments.

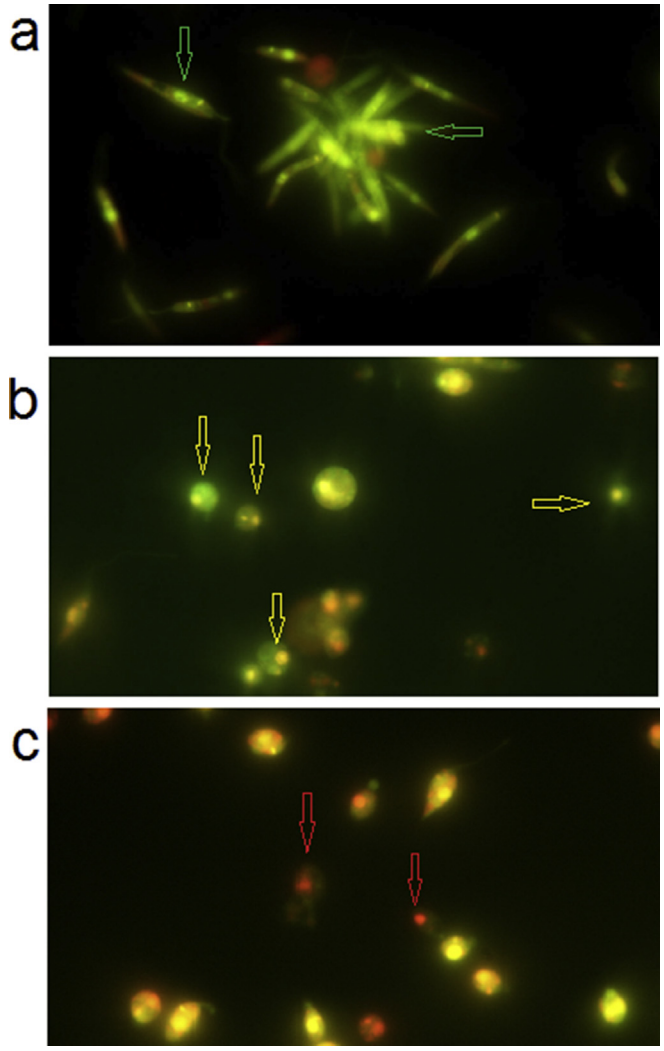


Fig. 6. Morphologic changes observed in *Leishmania infantum* after exposure to compounds **3** and **11**. Live parasites were determined by the uptake of acridine orange (green fluorescence) and exclusion of ethidium bromide (red fluorescence). Dead parasites was determined by the uptake of ethidium bromide (red fluorescence). (a) Control; (b) promastigotes exposed to 10 μg/mL of compound **3** for 48 h; c) promastigotes exposed to 10 μg/mL of compound **11** for 48 h. Apoptotic parasites show a loss of volume and nuclear condensation. Green arrows indicate living parasites, yellow arrow early apoptotic parasites and red arrow late apoptotic parasites. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.6. Cell cycle

The effects of **3** and **11** on cell cycle distribution was analyzed in *Leishmania infantum* promastigotes. Cells were cultured in presence of 10 μg/mL of each compound for 24 h. Flow cytometric analysis of cell cycle was carried out after staining of parasites with propidium iodide. Fig. 8a illustrates histogram of propidium iodide-stained log phase promastigotes. As shown, 51% of the growing parasite population is in G1, 30% in S, and 19% in the G2/M phase. The resemblance of the DNA distribution pattern indicates that parasites are stably growing populations. *Trans*-stilbene derivative **3** induced the arrest of *Leishmania* promastigotes prevalently in G2/M phase (Fig. 8b) while the terphenyl compound **11** in G1 phase of cell cycle (Fig. 8c). Cell cycle analysis depicts the effect of different treatments on progression of cell cycle wherein presence of cells in sub G0–G1 phase indicates the occurrence of apoptosis. Compounds **3** and **11** triggered apoptosis in *Leishmania infantum* promastigotes with an increase in sub G0–G1 population to 21.5%, and 32.6% respectively in comparison to untreated parasite control where only 6% cells were found to be apoptotic.

4. Discussion

Recently, several studies have shown that stilbene based compounds are endowed with antileishmanial activity (del Rey et al., 1999; del Olmo et al., 2001; Getti et al., 2006; de Almeida Machado et al., 2015; Fuchino et al., 2013). Since these molecules are generally non toxic and well tolerated *in vivo*, they may represent an interesting alternative to the currently antileishmanial therapy (Baur and Sinclair, 2006; Kee et al., 2014; Li et al., 2014). In a previous work, we studied a series of *cis*- and *trans*-resveratrol bearing 3,5-dimetoxy motif at the A phenyl ring and amino, methoxy and hydroxyl function at 3′- and/or 4′-position. **TTAS** was the most active stilbene showing in *Leishmania infantum* a IC_{50} value of 2.6 μg/mL, comparable to that of pentavalent antimonial sodium stibogluconate (pentostam) that we used as a reference compound (Tolomeo et al., 2013). To further explore the SAR of this class of compounds we studied herein a second series of *trans*-stilbenes 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 (**2**–**5**, Fig. 1, Table 1). Additionally, in an attempt to increase the chemical diversity of the compounds we studied a series of terphenyl derivatives (**6**–**12**, Fig. 1, Table 1) that notably do not bear the ethylene double bond that is the main reason for the chemical and metabolic instability of the stilbene derivatives (Metzler and Neumann, 1977; Metzler, 1975).

Initially all the compounds were tested *in vitro* against *Leishmania infantum* promastigotes in comparison with pentostam and with **TTAS** and resveratrol as reference compounds (Table 1). All stilbenes studied in this work (**2**–**5**, Table 1) were endowed with antileishmanial activity. Compound **3**, lacking the 3′-amino group was the most active showing on promastigotes a leishmanicidal activity comparable to pentostam and the parent **TTAS**, suggesting that the 3′-amino function is not necessary for the activity. Differently when the 4′-methoxy function of **TTAS** was replaced by the amino group as in compound **2** the activity decreased. A detrimental effect on the potency was also obtained with stilbenes **4** e **5** bearing an hydroxy group at C2′ and a methoxy function at C-4′ or C-3′ respectively.

Among the terphenyl series the range of activity was very wide. Derivatives, **6**, **8,** and **12**, which may be considered resveratrol analogues, showed an IC_{50} higher than 50 μg/mL. Terphenyls **7**, **9** and **11,** analogues of pterostilbene (IC_{50} 7.3 μg/mL) (Tolomeo et al., 2013) maintained the anti-leishmanial activity showing an IC_{50} of 5.3, 3.8 and 1.4 μg/mL respectively. Terpenyl **11** was the most potent compound of the series displaying an antileishmanial activity on promastigotes higher than the parent stilbene **3, TTAS** and pentostam. Therefore, these results indicate that the introduction of methoxy group at the stilbene resveratrol motif and to its terphenyl analogues seems to be important to confer a good antileishmanial activity to this class of compounds; in fact, in our cases the methoxy derivatives were more active than the corresponding phenols (compare **3** vs resveratrol, **11** vs **12**).

Compound **11** was highly active in amastigotes and in intramacrophagic amastigotes but was no toxic in normal cells and in macrophages. Of interest, a treatment with 10 μg/mL compound **11** for 48 h was able to eliminate completely intramacrophagic amastigotes without inducing any cytotoxicity on macrophages.

Cell cycle analysis showed that **11** causes an arrest of parasites in G1 phase of cell cycle while compound **3** in G2/M. Moreover, an

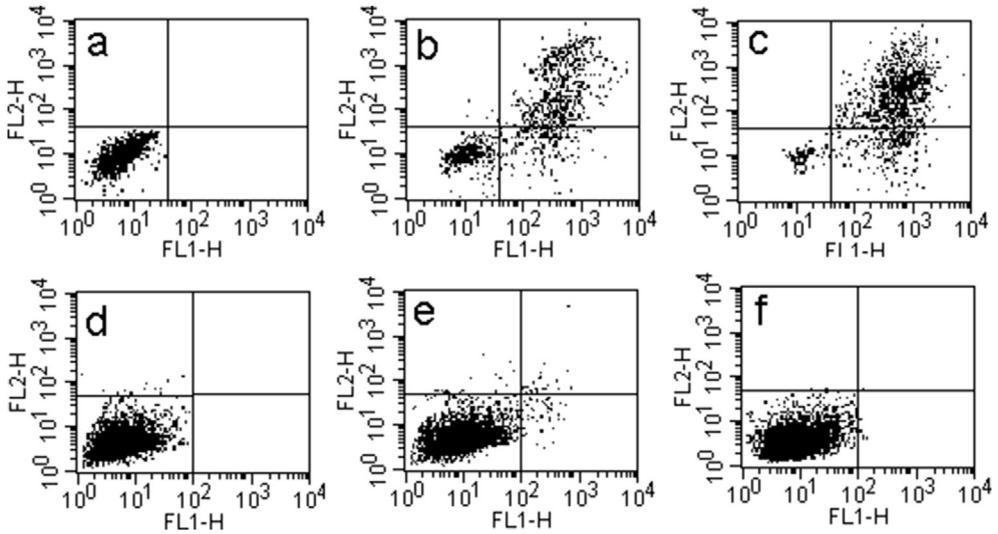


Fig. 7. Flow cytometry analysis of *Leishmania infantum* promastigotes (a–c) and U937 macrophage cells (d–f) following treatment with 10 μg/mL of compound **3** (b, e) and compound **11** (c, f) for 48 h. Control cells, parasites (a) and macrophages (d), were not exposed to the compounds. The cells were stained with Annexin V and Propidium iodide (PI). Lower left quadrant belongs to control cells (Annexin V negative/PI negative), lower right quadrant belongs to early apoptotic cells (Annexin V positive/PI negative), upper right quadrant belongs to late apoptotic cells (Annexin V positive/PI positive), upper left quadrant belongs to necrotic cells (Annexin V negative/PI positive). Data are representative of three separate experiments.

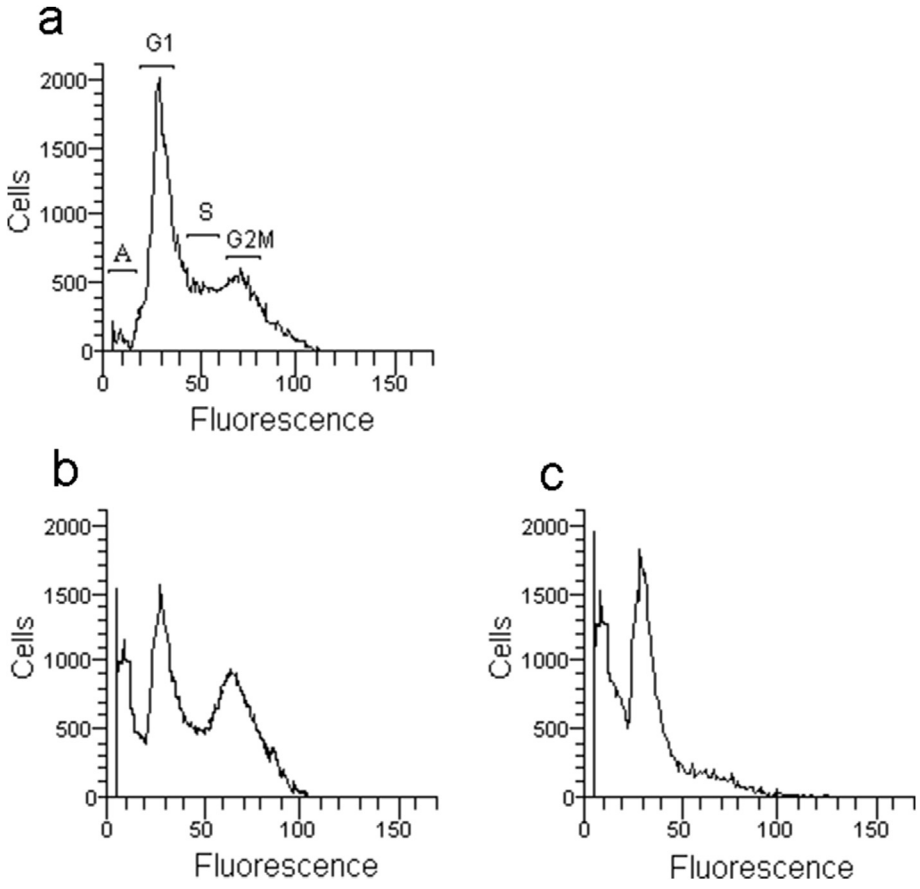


Fig. 8. Effects of compound **3** and **11** on DNA content/parasite following treatment of promastigotes for 24 h. The parasites were cultured without compound (control) (a) or with 8 μg/mL compound **3** (b) and **11** (c). Cell cycle distribution was analyzed by the standard propidium iodide procedure. Sub-G1 (A), G1, S, and G2–M cells are indicated in (a). Data are representative of three separate experiments.

apototic sub-G1 peak was observed in both samples treated with **11** and **3**, although the percentage of cells in sub-G1 was higher in the sample treated with **11**. Apoptosis was confirmed by morphological examination and Annexin V test; in both the percentage of apoptosis was higher in the samples treated with compound **11**. In contrast, no apoptotic effects were observed in human

macrophages. Apoptosis is an irreversible cell death process and drugs able to activate apoptosis can eliminate definitively parasites or cells of multicellular organisms. The ability of **11** and **3** to induce apoptosis in *Leishmanias* saving macrophages confirm the selective activity of these compounds.

In conclusion, we firstly demonstrated that compounds belonging to the class of terphenyls, as well as stilbene compouds, can be endowed with antileishmanial activity. This activity was potentiated by the introduction of methoxy groups. Terphenyl **11** was the most active compound displaying a leishmanicidal activity greater than stilbenes and pentostam. The ability of **11** to induce selectively apoptosis in parasites while saving macrophagic cells and its low toxicity on normal cells makes this compound interesting for further clinical and biological studies.

Conflict of interest

None.

Acknowledgments

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