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Article

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Discovery and pharmacological studies of 4-hydroxyphenyl-derived phosphonium salts active in a mouse model of visceral leishmaniasis

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KEYWORDS. Leishmania donovani, Leishmania infantum, phosphonium salt, in vitro activity, in vivo activity, pharmacokinetics, mechanism of action.

Abstract

We report the discovery of new 4-hydroxyphenyl phosphonium salt derivatives active in the submicromolar range (EC_{50} from 0.04 to 0.28 μM, SI > 10) against the protozoan parasite *Leishmania donovani.* The pharmacokinetics and *in vivo* oral efficacy of compound **1** [(16-(2,4-dihydroxyphenyl)-16-oxohexadecyl)triphenylphosphonium bromide] in a mouse model of visceral leishmaniasis were established. Compound **1** reduced the parasite load in spleen (98.9%) and liver (95.3%) of infected mice after an oral dosage of 4 daily doses of 1.5 mg/kg. Mode of action studies showed that compound **1** diffuses across the plasma membrane, as designed, and targets the mitochondrion of *Leishmania* parasites. Disruption of the energetic metabolism, with a decrease of intracellular ATP levels as well as mitochondrial depolarization together with a significant ROS production, contributes to the leishmanicidal effect of **1**. Importantly, this compound was equally effective against antimonials and miltefosine-resistant clinical isolates of *Leishmania infantum* indicating its potential as antileishmanial lead.

Introduction

Leishmaniasis is a neglected parasitic disease caused by parasites of the genus *Leishmania*. The clinical manifestations range from the (often self-healing) cutaneous form to the deadly visceral form caused by *Leishmania donovani* and *L.infantum*. With an estimated 700,000 to 1 million new cases and some 26,000 to 65,000 deaths occurring every year, leishmaniasis continues to be a menace in countries across the globe.[1] The epidemiology and spreading of this devastating disease, which was mainly distributed in tropical and subtropical areas, have been changing in the last years due to environmental, demographic, human behavioural factors and co-infections.[2] The lack of an anti-*Leishmania* vaccine and the toxicity of current drugs, combined with the rapid emergence of drug resistant *Leishmania* strains, represent major challenges for the control of this parasitosis. Current antileishmanial drugs are expensive and nonspecific, and usually require prolonged treatment regimens; as a result, high toxicity and low compliance are commonly observed. The increasing drug resistance and therapeutic failure reported and the reduced arsenal of drugs require the development of new compounds and treatment regimens that are safer, more effective and less vulnerable to the development and selection of drug resistant strains/isolates.

The cidal activity of benzyltriphenylphosphonium salts against trypanosomatid parasites was demonstrated 40 years ago by Kinnamon et al.[3-5] However, recently the interest for the antiparasitic activity of phosphonium salt derivatives rekindled.[6, 7] In recent years, we have synthesized several series of potent antiparasitic (bis)phosphonium salt derivatives that target the mitochondrion of kinetoplastid parasites *Trypanosoma brucei*[8] and *Leishmania.*[9] In *T. brucei*, the trypanocidal action of these compounds occurs by targeting the mitochondrial F_oF_1 ATPase[10] whereas in *Leishmania*, succinate dehydrogenase (complex II) appears to be the main target.[9] Lately, we have developed inhibitors of the trypanosome alternative oxidase (TAO) as potential chemotherapeutic agents against African trypanosomes.[11, 12] By applying a mitochondrion-targeting strategy using lipophilic cations (e.g. triphenylphosphonium salts, TPP) we were able to dramatically improve drug targeting and trypanocidal activity while retaining potency against the target protein.[13, 14]

TAO is not a validated target of *Leishmania* because the amastigote stage causing the disease in humans has a fully functional respiratory pathway, in contrast to bloodstream

form (BSF) trypanosomes which lack the canonical oxidative phosphorylation processes and rely exclusively on TAO for respiration.[15] However, based on the literature reports and our own experience, we envisaged that our mitochondrion-targeted (TAO inhibitor) compounds may have chemotherapeutic potential against *Leishmania* parasites. Accordingly, we carried out a focused screening of an in-house series of TAO inhibitors against *L. donovani* parasites which resulted in the discovery of potent leishmanicides (Table S1). As TAO is not a validated target in *Leishmania*, we set about to discover the mechanism of action (MoA) of these mitochondrion-directed compounds. As the most active and selective compounds presented potential metabolic instability, new derivatives were synthesized and tested against promastigote and amastigote forms of *L. donovani* (Chart 1).

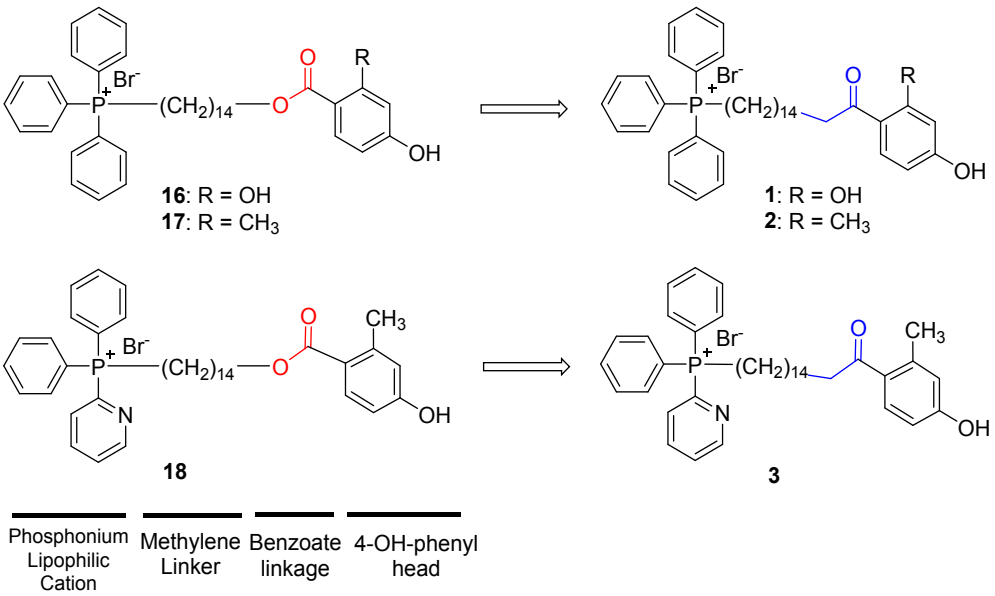
We report here the discovery of new phosphonium salts active against *L. donovani in vitro*, pilot preclinical pharmacological studies and strong *in vivo* activity in a mouse model of visceral leishmaniasis with an exploration of uptake and mechanism of action of lead compound **1**.

RESULTS

Preliminary screening and SAR studies. Thirty TAO inhibitors[11, 12, 14] were screened *in vitro* against promastigotes and the human-relevant intracellular amastigote forms of *L. donovani* HU3, and their cytotoxicity against MRC-5 or THP-1 cells was determined (Table S1). Eleven cationic compounds showed IC_{50} < 1 μM against intracellular amastigotes, among which eight were phosphonium salts (**15**–**18**, **23**–**24**, **26**, **31**) with selectivity indices (SI) versus mammalian cells ranging from 2 to 27. In general, the quinolinium salt derivatives were more cytotoxic and only three compounds (**10**, **11**, **32**) presented a fair activity/selectivity profile (IC_{50} ≈ 0.8 μM, SI = 3.7–5). Remarkably, compounds with a C14 methylene chain were slightly more selective towards *Leishmania* than shorter analogues with C10 or C12 (compare **10**/**11**, **15**/**16**, **29**/**30**). Three phosphonium hit compounds displaying EC_{50} < 0.2 μM against promastigotes and intracellular amastigotes of *L. donovani* and satisfactory selectivities (SI > 10) were selected as possible leads for *in vivo* studies (Table S1 and Chart 1: **16**, **17**, and **18**). Since the selected compounds contain an ester bond that can be hydrolyzed by serum hydrolases (e.g. the half-life of compound **16** is 1 h in mouse serum[12]), which may potentially limit

their *in vivo* efficacy, we decided to change the ester bond for a more metabolically stable keto link between the phenyl ring and the methylene chain (compounds **1**–**3**, Chart 1). The newly synthesized keto compounds were tested against promastigote and intracellular amastigote forms of *L. donovani* (Table 1), and the most promising lead compound (**1**) was selected for *in vivo* assays in a mouse model of visceral leishmaniasis. Pharmacokinetics, MoA and uptake studies were carried out. Besides, the trinaphthylphosphonium analogue (**4**) of compound **1** was synthesized as fluorescent probe for MoA studies whereas the aliphatic derivative **9** was used as control for SAR studies (Scheme 1).

Chart 1. Benzoate hit compounds active *in vitro* against *L. donovani* (**16**–**18**) and new keto analogues being studied (**1**–**3**)

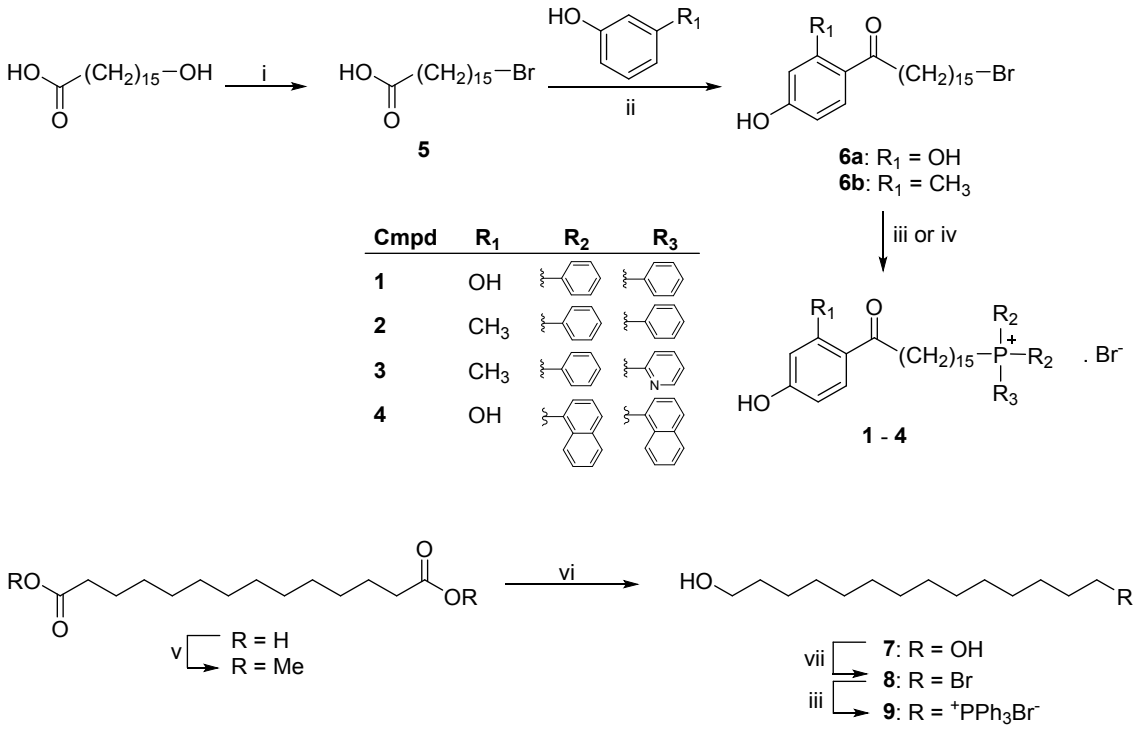


Chemistry. The phosphonium salt derivatives **1**–**4** were synthesized by nucleophilic substitution of the bromoalkyl precursors **6** with triphenylphosphine (**1**, **2**), diphenyl-2-pyridylphosphine (**3**), and tris(naphthalen-1-yl)phosphane (**4**), respectively (Scheme 1).

Compounds **6a** and **6b** were obtained by acylation of resorcinol and m-cresol with 16-bromohexadecanoic acid **5**, respectively. Compound **9** was synthesized by reaction of 14-bromotetradecanol **8** with Ph_3P in CH_3CN at 80 ºC. The 14-bromotetradecanol precursor

8 was synthesized in 3 steps from tetradecanedioic acid following reported procedures.[16, 17]

Scheme 1. Synthesis of compounds **1**–**4** and **9**



[a]Reagents and conditions: (i) HBr 48%, 110 ºC; (ii) BF_3-Et_2O, 110 ºC; (iii) triphenylphosphine (for **1** and **2**) or diphenyl-2-pyridylphosphine (for **3**), CH_3CN, 80 ºC, 5-18 days; (iv) tris(naphthalen-1-yl)phosphane (for **4**), DMF, 110 ºC, 5 days; (v) $SOCl_2$, MeOH, rt; (vi) $LiAlH_4$, THF, reflux; (vii) HBr 48%, cyclohexane, reflux.

Biology

In vitro* activity against promastigotes and amastigotes of *L. donovani. New keto derivatives **1**–**3** inhibited the growth of *L. donovani* promastigotes and intracellular amastigotes with submicromolar EC_{50} values similar to those of the ester counterparts **16**–**18** (Table 1). Remarkably, the leishmanicidal activity of **1**–**3** against intracellular amastigote forms (EC_{50} = 0.1 μM) was twice as good as the control drug amphotericin B (AmB) (EC_{50} = 0.24 μM). The cytotoxicity of **1**–**3** against THP-1 cells was comparable or slightly lower than the ester analogues **16**–**18**, resulting in higher selectivity for the new compounds (SI >20).

Based on the promising *in vitro* activity/selectivity profile of the new keto derivatives against intracellular amastigotes of *L. donovani*, which is the human-relevant form of the parasite, compound **1** (EC_{50} = 90 nM, SI = 31.2) was selected as lead for further pharmacological studies.

Table 1. Activity of 4-hydroxyphenyl keto analogues **1**–**3** and ester counterparts **16**–**18** in promastigote and intracellular amastigote forms of *L. donovani* HU3 and cytotoxicity against THP-1 cells[a]

Cmpd	**EC_{50}(μM)**			**SI**[b]
	L. donovani promastigotes	*L. donovani* intracellular amastigotes	THP-1 cells	
1	0.07 ± 0.02	0.09 ± 0.02	2.81 ± 0.35	31.2
2	0.09 ± 0.01	0.10 ± 0.01	2.45 ± 0.41	24.5
3	0.28 ± 0.05	0.10 ± 0.02	2.11 ± 0.12	21.1
4	3.54 ± 0.36[c]	1.25 ± 0.22[c]	12.85 ± 2.30	10.3
16	0.04 ± 0.01	0.11 ± 0.02	3.02 ± 0.47	27.6
17	0.09 ± 0.02	0.15 ± 0.01	1.84 ± 0.22	12.3
18	0.18 ± 0.02	0.12 ± 0.03	2.35 ± 0.38	19.6
9	0.33 ± 0.04	0.35 ± 0.05	3.05 ± 0.61	8.7
AmB[d]	0.13 ± 0.02	0.24 ± 0.06	27.85 ± 3.50	116
MIL[e]	6.51 ± 0.35	0.91 ± 0.05	26.25 ± 2.58	28.8

[a] Parasites and THP-1 cells were grown in the presence of increasing concentrations of compounds. Data are means ± standard deviations from three independent experiments. [b] SI= Selectivity Index (EC_{50} THP-1 / EC_{50} parasite (amastigote forms)). [c] Compound tested in *L. infantum*. [d] AmB: amphotericin B. [e] MIL: Miltefosine.

Activity of compound 1 against resistant clinical isolates of *L. infantum.* The increasing therapeutic failure and appearance of drug resistance represents a serious limitation of current antileishmanial chemotherapy ,[18-22] hence we assessed the sensitivity of compound **1** in both promastigote and intracellular amastigote forms of resistant lines

of *L. infantum* obtained from clinical cases.[23] No significant differences in sensitivity to compound **1** were found between the drug sensitive reference line (LEM3049) and *L. infantum* LEM 3323 (resistant to Sb^{III})[24], *L. infantum* LEM 5159 (resistant to miltefosine)[24] and *L. infantum* LEM 2126 (resistant to miltefosine and paramomycin)[25] (Table 2).

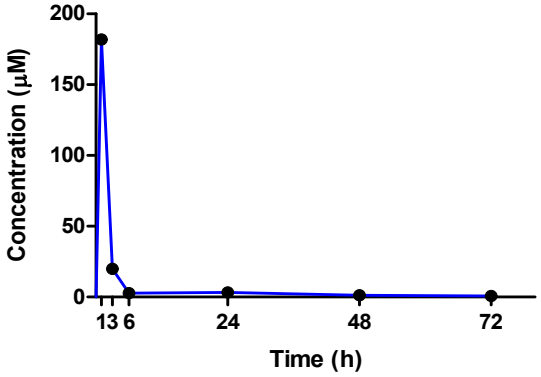
Table 2. Leishmanicidal activity of compound **1** on promastigote and intracellular amastigote forms of clinical isolates of *L. infantum*[a]

	EC_{50}(μM)			
	Promastigotes		Intracellular amastigotes	
L. infantum	Cmpd **1**	AmB	Cmpd **1**	AmB
LEM3049	0.08 ± 0.03	0.06 ± 0.02	0.12 ± 0.06	0.17 ± 0.02
LEM3323	0.10 ± 0.02	0.07 ± 0.03	0.15 ± 0.07	0.18 ± 0.02
LEM5159	0.11 ± 0.02	0.05 ± 0.01	0.14 ± 0.02	0.16 ± 0.03
LLM2126	0.12 ± 0.03	0.10 ± 0.02	0.14 ± 0.01	0.16 ± 0.01

[a]Parasites were grown as described in Experimental section, in the presence of increasing concentrations of compounds. *L. infantum* clinical isolates used: LEM 3049 line as a reference line; LEM 3323 a clinical line resistant to Sb^{III}; LEM 5159 a clinical line resistant to miltefosine (MIL) and LEM 2126 a clinical line resistant to MIL and paromomycin. Amphotericin B (AmB) was used as a reference drug for *Leishmania*. Data are means ± standard deviation from three independent experiments.

Preliminary pharmacokinetic studies of compound 1. A snapshot pharmacokinetic study was performed with male BALB/c mice to determine the oral bioavailability of compound **1**. Since the molecule is hardly soluble in water, it was formulated in 8% DMSO in water and a single dosage of 20 mg/kg, corresponding to 500 μg of compound **1**, was given orally (n = 3 mice). The maximum plasma concentration (C_{max} = 182 μM) was reached rapidly 1 h after oral dosage (Figure 1, Table S2). The compound showed an apparent multi compartmental behavior with a rapid elimination from the plasma 6 h after treatment ($t_{1/2}$ = 1 h) and slower elimination in the 6–72 h post-treatment ($t_{1/2\ apparent}$ = 23 h). Taking into account the PK data and the fact that signs of acute toxicity were observed at the 20 mg/kg dosage, a 16-fold lower dose (1.5 mg/kg) and different vehicle (i.e. γ-cyclodextrin) was selected for in vivo efficacy studies.

Figure 1. Plasma concentration of compound **1** obtained after oral administration of 20 mg/kg of compound **1** in 5-8% DMSO to BALB/c mice. Values correspond to the determination in pooled samples (n=3).



Pharmacokinetic parameters	
T_{max}	1 h
C_{max}	182 µM
$t_{1/2}$	
First phase (< 6 h)	1 h
Second phase (6–72 h)	23 h

Microsomal stability. The microsomal stability of **1** toward metabolism by cytochrome P450 (Phase-I metabolism) and Uridine Glucuronosyl-Transferase (UGT) (Phase-II metabolism) was studied in presence of NADPH and UDPGA. Compound **1** was rapidly metabolized by mouse (CD-1) liver microsomes (high intrinsic clearance) with half-life < 30 min, confirming the PK data observed in vivo. In contrast, **1** was metabolized slowly by human liver microsomes (low to moderate intrinsic clearance) with $t_{1/2} > 2$ h, and very slowly by human liver S9 fraction within 2 h of reaction (Table S3) suggesting species-related differences. In comparison, high intrinsic clearance of the control drug diclofenac by human fractions was observed under the same conditions ($t_{1/2} < 30$ min).

***In vivo* efficacy assay in BALB/c mouse model of visceral leishmaniasis**. Compound **1** was formulated as a colloidal suspension in 5% γ-cyclodextrin in water (w/w); the thermodynamic solubility of **1** in this vehicle was measured as 520 ± 10 mg/L. Based on the pharmacokinetics, stability and absorption data, compound **1** was administered orally in the *L. infantum* BALB/c mouse model at 1.5 mg/kg for 4 days. We established three groups of mice (five mice per group): (i) G1, non-infected mice + compound **1**; (ii) G2,

infected mice + vehicle, and (iii) G3, infected mice + compound **1**. The results are expressed as mean number of amastigotes per mg of tissue. Amastigote burdens in the liver and spleen were determined after 48 h of the end of treatment with compound **1**. The vehicle-treated controls showed high amastigote burdens in the spleen (607.3 ± 177.0) and lower burdens in the liver (4.92 ± 1.21) and we observed a reduction of 98.9% (6.47 ± 1.77) and 95.3% (0.18 ± 0.07), respectively in the infected mice treated with compound **1** (Table 3).

Table 3. Activity of compound **1** in the *L. infantum* BALB/c mouse model with respect to organ burden and efficacy (percentage of reduction)

	Amastigote burden			
	Liver		**Spleen**	
Dosing group	**Mean[a] + SD**	**% reduction**	**Mean + SD**	**% reduction**
G1 (Non-infected + Cmpd **1** at 1.5 mg/kg p.o.[b] for 4 days)	-	-	-	-
G2 (Infected + vehicle [5% γ-cyclodextrin])	4.92 ± 1.21	-	607.25 ± 176.96	-
G3 (Infected + Cmpd **1** at 1.5 mg/kg p.o. for 4 days)	0.18 ± 0.07	95.3	6.47 ± 1.77	98.9

[a]Mean number of amastigotes per mg of tissue ± SD. [b]p.o., orally.

Pharmacokinetics and biodistribution. Compound **1** was only detected in plasma samples corresponding to the first sampling (after the 4 days of treatment) (Table S4). Notably, all the plasma concentrations were well over the EC_{50} value measured for *L. donovani* (0.09 μM). In all other cases (solid organs and plasma 2 days after the end of treatment) the concentration of **1** was below the detection limit of the HPLC technique. Absence of detection of **1** in the samples obtained 2 days after the end of the treatment is consistent with the rapid clearance observed in the snapshot experiments and with

microsomal fractions in vitro. Hence, the absence of detection of **1** in the solid organs analyzed (liver, spleen) could be due to the elimination of the molecule in target organs.

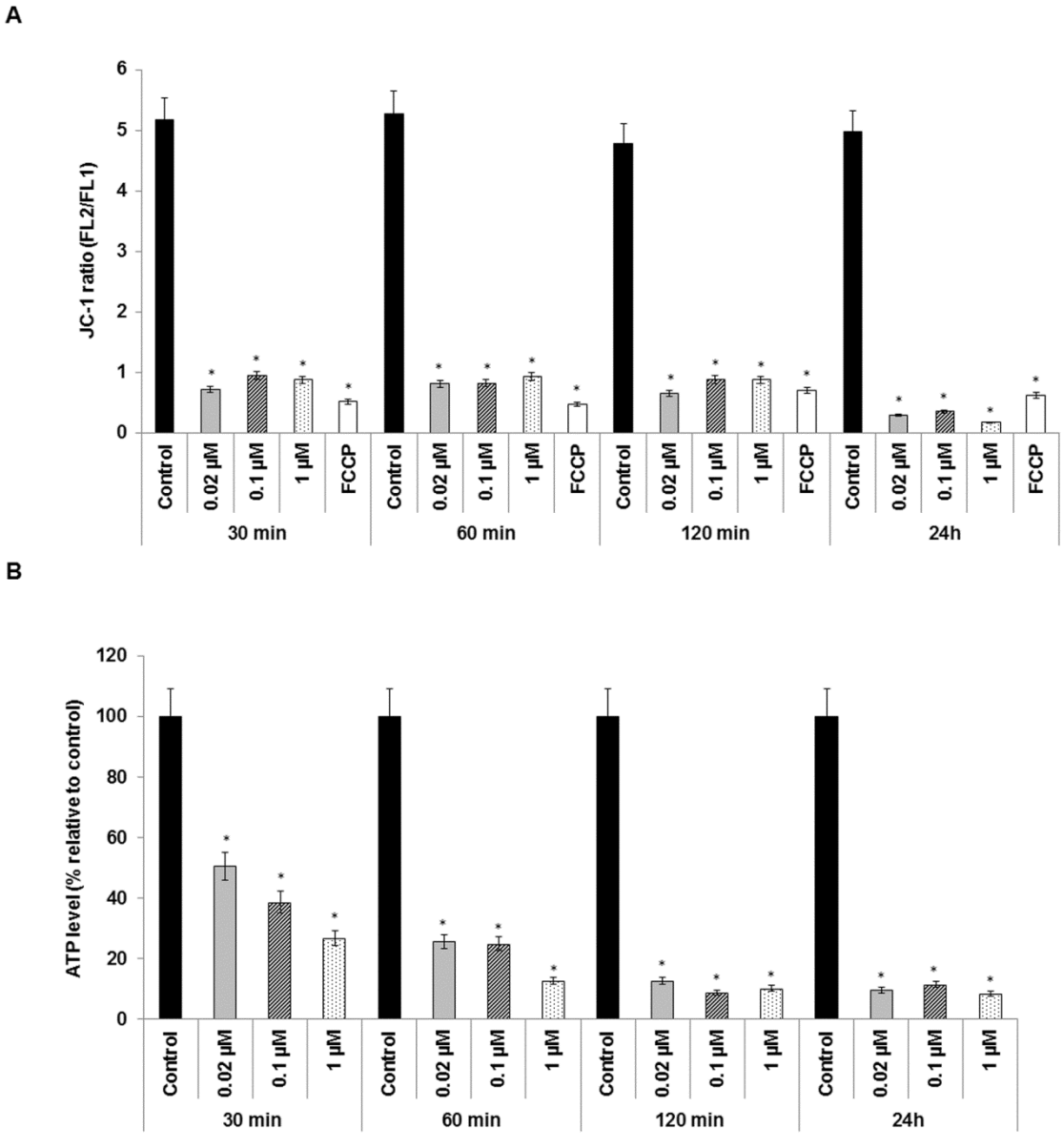
Mode of action studies: uptake and effect of compound 1 on mitochondrial membrane potential ($\Delta\Psi_m$), ATP levels, reactive oxygen species (ROS), plasma membrane integrity and DNA fragmentation of *L. infantum* parasites.

Triphenylphosphonium salt derivatives are cationic molecules with a delocalized charge and enhanced hydrophobic character; these properties allow their diffusion across biological membranes driven by the electrochemical potential, resulting in their accumulation in organelles with high membrane potential such as mitochondria.[8-10, 26]

To directly measure the cytotoxicity of compound **1**, we studied if the mitochondrial function of *Leishmania* was affected by the molecule. The effect of compound **1** on the mitochondrial membrane potential ($\Delta\Psi_m$) of *L. infantum* promastigotes was determined using the JC-1 fluorescence marker. We observed a fast depolarization of $\Delta\Psi_m$ from the first concentration (5 times under EC_{50}) and time-point tested (0.02 μM, 30 min) with a decrease of 86%, showing a level of $\Delta\Psi_m$ similar to the classical uncoupling reagent FCCP, used at 10 μM for 30 min (Figure 2A). The maximum decrease in $\Delta\Psi_m$ was observed after 24 h at 1 μM of compound **1** (97%) with visible signs of cellular death in the culture of *L. infantum* parasites. These results strongly suggest that compound **1** affects the mitochondrion.

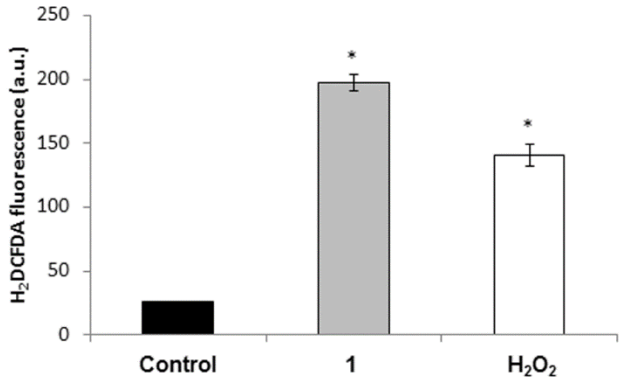
Bearing in mind that $\Delta\Psi_m$ is essential for mitochondrial ATP synthesis,[27] we analyzed the levels of ATP in parasites treated with 0.02, 0.1 and 1 μM of **1** after 30, 60, 120 min and 24 h using the bioluminescence assay CellTiter-Glo. Compound **1** elicited a rapid time and concentration dependent depletion of ATP in the treated parasites (Figure 2B). In the first 30 min, a dose-dependent decrease of 50–74% of ATP levels was observed upon treatment with **1**. The maximum decrease of ATP levels (91, 89 and 87% after incubation with 0.02, 0.1 and 1 μM of **1**, respectively) was reached after 120 min (Figure 2B).

Figure 2. Altered mitochondrial membrane potential and ATP levels in *L. infantum* lines exposed to compound 1. *L. infantum* line was preincubated with 0.02, 0.1 and 1 µM of **1** for 30 min, 60 min, 120 min, and 24 h. (A) To determine $\Delta\Psi_m$, parasites were incubated with 5 µM JC-1 for 10 min, as described in the Experimental section. The FL2/FL1 fluorescence ratio was measured by flow cytometry analysis. Parasites were pretreated with 10 µM FCCP for depolarization control. The fluorescence intensity was determined by flow cytometry analysis and expressed as Relative Fluorescence Units (RFU). (B) To analyze ATP levels, a 25 µL aliquot of parasites was transferred to a 96-well plate and mixed with the same volume of CellTiter-Glo (Promega) for measurement of the sample bioluminescence. Data are the means ± SD of at least three independent experiments. Significant differences versus the control line were determined by Student's t-test (*: $p < 0.01$).



Several antileishmanial drugs have been described to induce cell death mediated by the accumulation of ROS (Reactive Oxygen Species) in the mitochondria of parasites.[28] Taking into account the relationship between the decrease of $\Delta\Psi_m$ induced in *Leishmania* by different drug treatments[29] and ROS production, we studied ROS levels in parasites treated with 1 μM of compound **1** for 2 h using the intracellular probe H_2DCFDA. A significant increase (8-fold) of ROS levels was observed compared with the untreated control parasites. This effect was even higher than that of H_2O_2 (100 μM, 15 min) used as positive control for the generation of ROS (Figure 3). Consequently, the generation of ROS after treatment with **1** could contribute to the leishmanicidal effect of the compound.

Figure 3. ROS levels in *L. infantum* lines exposed to compound 1. Intracellular ROS levels were measured using the fluorescent probe H_2DCFDA. *L. infantum* line was incubated with 1 μM of compound **1** for 2 h, as described in the Experimental section. After treatment, parasites were incubated with 40 nM H_2DCFDA for 30 min at 28 ºC. The fluorescence intensity was determined by flow cytometry analysis and expressed as Relative Fluorescence Units (RFU). We used H_2O_2 (100 μM, 15 min) as positive control of generation of ROS. Data are the means ± SD of at least three independent experiments. Significant differences versus the control line were determined by Student's t-test (*: $p < 0.01$).



One of the mechanisms involved in cell death which usually acts together with the loss of $\Delta\Psi_m$, ATP depletion and generation of ROS, is the alteration of plasma membrane permeability. Hence, we studied this parameter by the increase in fluorescence of SYTOX green, a membrane impermeable intercalating agent. Permeabilized promastigotes with 1% Triton-X100 (TX100) were used as a positive control. Incubation of *Leishmania* parasites with 0.02, 0.1 and 1 μM of compound **1** did not affect the plasma membrane

permeability significantly (Figure 4). Additionally, depolarization of the plasma membrane was measured using $DiBAC_4(3)$ as a fluorescence probe. After treatment with 0.02, 0.1 and 1 μM of compound **1** for 2 h, we observed significant changes in the plasma membrane potential in a concentration-dependent manner (Fig. 4B). Thus, compound **1** does not induce osmolysis as it enters the parasite by depolarizing the plasma membrane without inducing pore formation.

Finally, the DNA fragmentation, typical of apoptotic processes, was tested after treatment with compound **1** using the TUNEL assay. Parasites incubated with different concentrations of compound **1** (0.02, 0.1, 1 and 5 μM) for 4 h were TUNEL negative (Fig. 5A). Additionally, necrotic cell death was analyzed by parasite staining with propidium iodide (PI). We observed PI positive parasites in all concentrations tested (Fig. 5B). Consequently, the cell death produced by the treatment with compound **1** is not related with the apoptotic process.

Figure 4. Plasma membrane permeability and membrane potential in *L. infantum* lines exposed to compound 1. *L. infantum* line was incubated with 0.02, 0.1 and 1 μM of **1** for 2 h, as described in the Experimental section. (A) After treatment, parasites were incubated with SYTOX Green for 15 min at 28 ºC. The fluorescence intensity was determined by flow cytometry analysis and expressed as Relative Fluorescence Units (RFU). Permeabilized promastigotes with 1% Triton-X100 (TX100) were used as a positive control. (B) After treatment, parasites were incubated with 2 μM DiBAC4(3) for 10 min at 28 °C. Parasites treated with a 10 μM concentration of the depolarizing agent carbonyl cyanide m-chlorophenylhydrazone (CCCP) for 15 min were used as controls. Data are the means ± SD of at least three independent experiments. Significant differences versus the control line were determined by Student's t-test (*: $p < 0.01$).

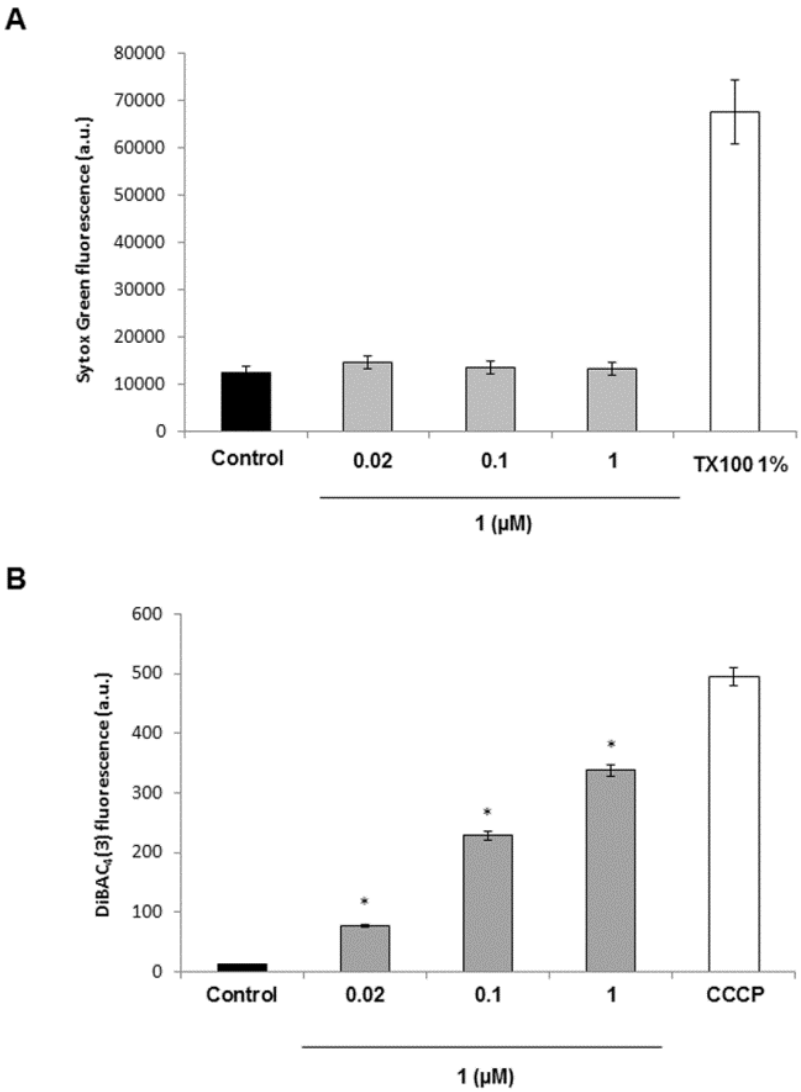
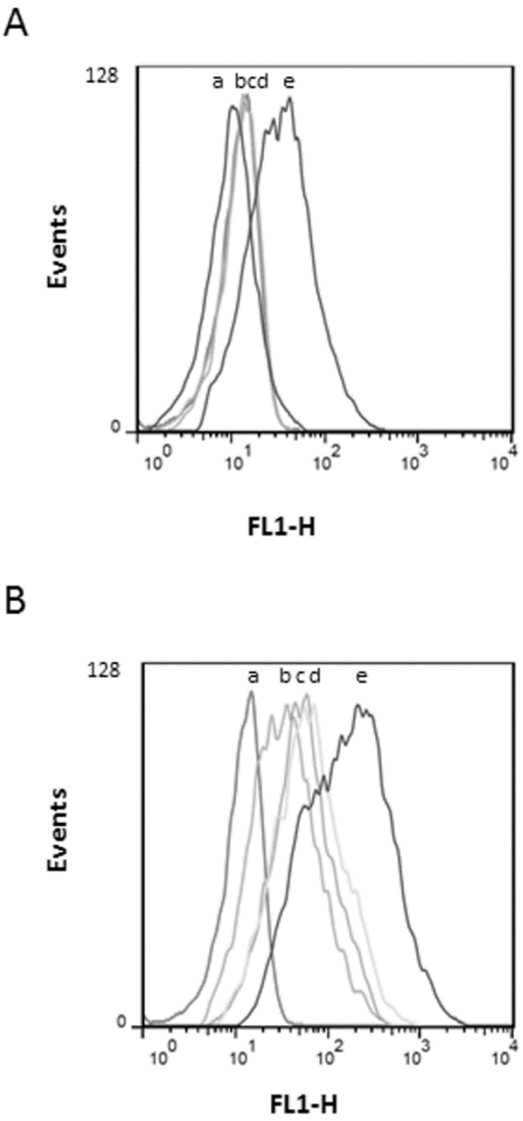
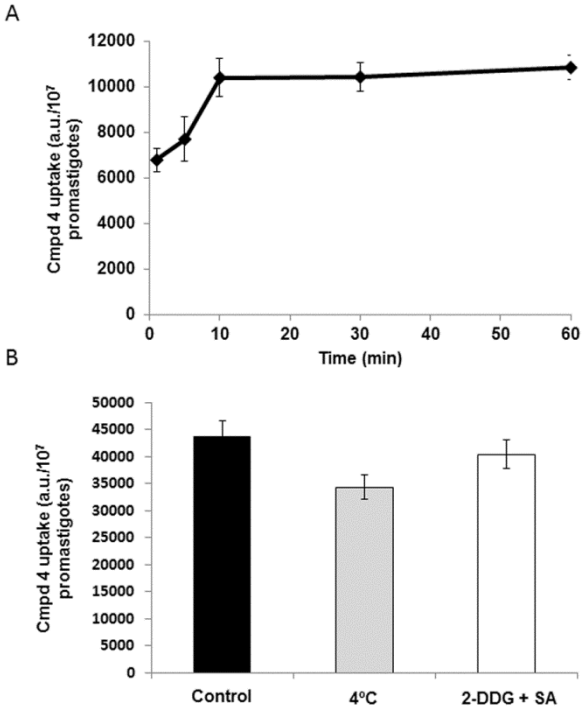


Figure 5. DNA fragmentation in *L. infantum* lines exposed to compound 1. Representative histogram of (A) TUNEL analysis and (B) propidium iodide (PI) labeling, respectively, of *L. infantum* promastigotes treated with compound **1**. Parasites were treated with different concentrations of compound **1**: 0.01 (b), 0.2 (c), 1 (d) and 5 (e) μM for 4 h at 28 °C, using untreated parasites (a) as controls. Fluorescein-dUTP and PI nucleic acid labeling were analyzed by flow cytometry as described in Experimental part. Histograms are representative of three independent experiments with 10,000 parasites analyzed per group.



Uptake of compound 4. To expand the knowledge about the mechanism of action of compound **1**, we used a fluorescent analogue (compound **4**) to study the uptake of this kind of TPP molecules. Considering the EC_{50} and the limit of the fluorescence signal obtained, we used 20 μM of compound **4** (Exc. 340 nm; Em. 510 nm). The uptake of compound **4** at 28 ºC was fast and reached saturation in about 10 min (Figure 6A). No differences in compound **4** uptake were observed in the ATP-depleted parasites or at 4 ºC (Figure 6B). Compound **4** showed fast entry kinetics into *Leishmania* parasites in an energy- and temperature-independent process, this suggesting plasma membrane diffusion process as expected for this class of lipophilic cations.

Figure 6. Uptake of compound 4 by *L. infantum* parasites. (A) Kinetics of compound **4** uptake at 28 ºC in promastigotes at different time-points (1, 5, 10, 30 and 60 min). (B) Energy and temperature dependence of compound **4** uptake. We used non-treated parasites (control, incubated at 28 ºC), treated with 2-deoxy-D-glucose plus NaN_3 (2DDG+SA) or parasites incubated at 4 ºC. Data are the means ± SD of three independent experiments.



DISCUSSION AND CONCLUSIONS

The mitochondrion of trypanosomatid parasites is a well-known target of cationic compounds.[9, 10, 14, 30] This is the case, for instance, for miltefosine and paromomycin, two of the currently used drugs against *Leishmania*.[31, 32] The TPP salt derivatives reported here are lipophilic cations that can accumulate in negatively charged organelles driven by the electrochemical potential, so their profound effect on *Leishmania* mitochondria was expected. These compounds are weak acids with pK_a values close to 7.4 (the measured pK_a value of compound **1** is 7.62 at 25 ºC in H_2O, see experimental section), meaning that approximately 50% of the molecule will exist as phenolate ion at physiological pH. Hence, 50% of molecule **1** will behave as electrically neutral zwitterion (logP = 0.38) whereas another 50% will behave as cation (log P = 1.17). These physicochemical properties of **1** may have an impact on the drug solubility and absorption, and possibly on its MoA. In fact, the logP value of cation **1**, that falls within the logP range of 1–3 for compounds considered to be highly permeable,[33] agrees with the observed oral bioavailability of compound **1** in vivo.

Our data show that compound **1** is taken up by *Leishmania* parasites by diffusion through the plasma membrane; in addition, we observed a depolarization of the plasma membrane that did not affect the integrity of parasites. Then, the compound acts on the mitochondrion affecting the energy metabolism with a depolarization of $\Delta\Psi_m$ that influences the ATP synthesis together with a significant ROS production. Cortes *et al*[6] have shown that related gallic acid–TPP conjugates with linkers of 10 and 12 methylene units, which are trypanocidal *in vitro* against *T. cruzi*, also exert an uncoupling effect (i.e. the positive charge of the TPP cation counteracts the negative charge at the level of the mitochondrial matrix) in *T. cruzi* trypomastigotes, although to a lower extent than the classical uncoupler FCCP. In contrast, compound **1** displayed a fast uncoupling effect as strong as the control drug FCCP in promastigotes of *L. infantum* at low concentration (5 times under EC_{50}). This collapse of $\Delta\Psi_m$ could be explained by the combined action of an uncoupling effect and respiratory chain inhibition, although plasma membrane permeabilization does not appear to be involved.[34]

To determine whether compound **1** produced apoptosis-like cell-death, which is characterized by DNA nicking and fragmentation as the final outcome, its effect on the DNA fragmentation was studied; our results were consistent with a non-programmed cell-death or necrosis.

The preliminary SAR studies obtained from the screening of compounds **10**–**40** show that the antileishmanial activity/selectivity profile depends on the substitution pattern of the 4-hydroxyphenyl head. Indeed, the lower efficacy (3- to 8-fold higher EC_{50} value) observed for the alkyl compound **9** lacking the (2-Me/2-OH)-4-hydroxy aromatic head, compared with **1**, **2**, **16**, and **17** agrees with these findings and warrants future SAR studies on this scaffold. Advantageously, the new keto analogues **1**–**4** synthesized in this work are metabolically more stable than the benzoate counterparts **16**–**18**[12] and they also proved to be slightly more selective (2- to 4-fold) towards *Leishmania*. Thus, lead compound **1** was relatively stable when challenged by phase I and phase II metabolism with human fractions, indicating favorable pharmacokinetic properties for future studies. At present, miltefosine is the only effective oral drug available among the limited arsenal of leishmanicidal treatments. The increasing appearance of drug resistance and therapeutic failure, together with the high cost of the current treatments, underscores the need to find new orally active drugs for this disease. The activity (EC_{50}) of compound **1** against promastigotes and intracellular amastigotes of *L. donovani* (0.07 and 0.09 μM, respectively) with a SI=31.2 compared well with those obtained for miltefosine (6.51 and 0.91 μM, respectively) with a SI=28.8. Furthermore, the results from the *in vivo* acute murine model showed a strong reduction of parasite burden in spleen and liver (98.9 and 95.3%, respectively) with an oral administration of 1.5 mg/kg of **1** for 4 days. Similar results in the reduction of parasite burden and efficacy have been published recently with a high dose of miltefosine (i.e. 40 mg/kg once a day for 5 days) in a similar mouse model of leishmaniasis,[35] this reinforcing the relevance of the efficacy results obtained with compound **1** at much lower dosage. The potential of compound **1** for the treatment of chronic leishmaniasis using hamster or dog models will be the subject of future studies.

In summary, the results disclosed here provide the evidence of significant antileishmanial activity of 4-hydroxyphenyl phosphonium derivatives *in vitro* and *in vivo* in a BALB/c mouse model. The highest activity and the best selectivity index were presented by compound **1**. In fact, the reduction observed in the parasite burden in target organs after treatment with compound **1** in our murine model is similar to that obtained with miltefosine with much higher doses. Exact MoA is unknown although the data obtained suggest favorable PK characteristics, a fast entry of these compounds by diffusion across the plasma membrane, alteration of the energy metabolism ($\Delta\Psi_m$, ATP), production of ROS and a necrosis. The determination of the specific target protein (complex) of

compound **1** in the mitochondrial electron transport chain will be approached in future works.

EXPERIMENTAL PART

Chemistry. Anhydrous solvents were purchased to ACROS Organics in AcroSeal® bottles and used as received. Thin Layer chromatography (TLC) was performed on silica gel 60 F254 aluminum TLC plates (MERCK). Chromatography was performed on silica gel 60 (0.040–0.063 mm, 230–400 mesh ASTM, MERCK). LC-MS spectra were recorded on a WATERS apparatus integrated with a HPLC separation module (2695), PDA detector (2996) and Micromass ZQ spectrometer using electrospray ionization (ES^+). Analytical HPLC was performed with a SunFire C18-3.5 μm column (4.6 mm × 50 mm). Mobile phase A: CH_3CN + 0.08% formic acid and B: H_2O + 0.05% formic acid. UV detection was carried over 190 to 440 nm. ^1H NMR and ^{13}C NMR spectra were registered on a Bruker Avance-300, Varian Inova-400, Varian-Mercury-400, and Varian-500. Chemical shifts of the ^1H NMR spectra were referenced to tetramethylsilane (δ 0 ppm) for Chloroform-*d* and the residual peak of the deuterated solvent (δ 3.31 ppm for Methanol-d_4) Chemical shifts of the ^{13}C NMR spectra were referenced to $CDCl_3$ (δ 77.16 ppm) and CD_3OD (δ 49.0 ppm). Coupling constants *J* are expressed in hertz (Hz). Accurate mass was measured with an Agilent Technologies Q-TOF 6520 spectrometer using electrospray ionization. All of the biologically tested compounds were ≥ 95% pure by HPLC.

(15-(2,4-Dihydroxyphenyl)-15-oxopentadecyl)triphenylphosphonium bromide (1). A Kimax tube was charged with **6a** (75 mg, 0.18 mmol) and Ph_3P (51 mg, 0.19 mmol), and flushed with argon. Anhydrous acetonitrile (1.5 mL) was added and the tube was screw-capped. The reaction mixture was stirred 18 days at 80 ºC. The tube was allowed to stand at 4 ºC for several days whereupon a precipitate settled at the bottom of the tube. The supernatant was discarded and a small volume of CH_3CN was added to the tube to rinse the precipitate. The tube was centrifuged and the supernatant was discarded. Rinsing of the precipitate was repeated with Et_2O following the same protocol. The pure product **1** was isolated as a film-like colorless solid (85 mg, 100%). HPLC >95%, ^1H NMR (400 MHz, Chloroform-*d*) δ 12.71 (s, 1H), 9.70 (s, 1H), 7.78 – 7.56 (m, 15H), 7.48 (d, *J* = 8.9 Hz, 1H), 6.55 (dd, *J* = 8.9, 2.4 Hz, 1H), 6.33 (d, *J* = 2.4 Hz, 1H), 3.41 (td, *J* = 13.1, 12.6,

7.3 Hz, 2H), 2.74 (t, *J* = 7.5 Hz, 2H), 1.60 (q, *J* = 7.4 Hz, 2H), 1.56 – 1.42 (m, 4H), 1.30 – 1.05 (m, 20H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 205.2, 165.3, 165.0, 135.3 (d, *J* = 3.0 Hz), 133.6 (d, *J* = 9.9 Hz), 132.2, 130.7 (d, *J* = 12.5 Hz), 118.2 (d, *J* = 86.0 Hz), 112.8, 108.7, 103.4, 37.9, 30.6 (d, *J* = 15.5 Hz), 29.5, 29.4, 29.3, 29.2, 29.14, 29.11, 29.08, 25.2 (d, *J* = 1.8 Hz), 22.8 (d, *J* = 50.3 Hz), 22.7 (d, *J* = 4.5 Hz). HRMS (ESI$^+$) *m/z* 609.3482 ($C_{40}H_{50}PO_3$ requires: 609.3498).

(16-(4-Hydroxy-2-methylphenyl)-16-oxohexadecyl)triphenylphosphonium bromide (2). A Kimax tube was charged with **6b** (6.9 mg, 0.016 mmol) and Ph_3P (4.2 mg, 0.016 mmol), and flushed with argon. Anhydrous acetonitrile (0.6 mL) was added and the tube was screw-capped. The reaction mixture was stirred 5 days at 80 ºC. The solvent was removed under vacuum and the product was isolated by precipitation from MeOH/Et_2O/hexane at 4 ºC overnight. The supernatant was removed and the precipitate was rinsed successively with Et_2O and hexane. Off-white gummy solid (7.6 mg, 69%). HPLC ≥ 95%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 12.36 (s, 1H), 7.84 – 7.74 (m, 6H), 7.72 (td, *J* = 7.3, 1.8 Hz, 3H), 7.63 (td, *J* = 7.7, 3.5 Hz, 6H), 7.57 (d, *J* = 8.2 Hz, 1H), 6.71 (d, *J* = 1.7 Hz, 1H), 6.63 (dd, *J* = 8.2, 1.7 Hz, 1H), 3.76 (dt, *J* = 13.2, 7.4 Hz, 2H), 2.87 (t, *J* = 7.5 Hz, 2H), 2.27 (s, 3H), 1.66 (p, *J* = 7.4 Hz, 2H), 1.60 – 1.50 (m, 2H), 1.38 – 1.07 (m, 22H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 206.6, 162.8, 147.9, 135.1 (d, *J* = 3.0 Hz), 133.9 (d, *J* = 10.0 Hz), 130.6 (d, *J* = 12.5 Hz), 130.1, 120.3, 118.7 (d, *J* = 85.6 Hz), 118.6, 117.3, 38.4, 30.6 (d, *J* = 15.5 Hz), 29.71, 29.69, 29.67, 29.64, 29.57, 29.54, 29.45, 29.41, 29.3, 24.8, 23.0 (d, *J* = 49.3 Hz), 22.8 (d, *J* = 4.6 Hz), 22.1. HRMS (ESI$^+$) *m/z* 607.3709 ($C_{41}H_{52}PO_2$ requires: 607.3705).

(16-(4-Hydroxy-2-methylphenyl)-16-oxohexadecyl)diphenyl(pyridin-2-yl)phosphonium bromide (3). Using the same procedure as above for compound **2**, a mixture of **6b** (5.7 mg, 0.014 mmol) and Ph_3P (3.7 mg, 0.014 mmol) in dry acetonitrile (0.6 mL) was stirred at 80 ºC for 5 days under argon atmosphere. The solvent was removed under vacuum and the product was isolated by precipitation from MeOH/Et_2O/hexane at 4 ºC overnight. The supernatant was removed and the precipitate was rinsed successively with Et_2O and hexane. Brownish gum (4.6 mg, 48%). HPLC ≥ 95%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 12.36 (s, 1H), 8.81 (dt, *J* = 4.7, 1.5 Hz, 1H), 8.42 (ddt, *J* = 7.9, 5.9, 1.1 Hz, 1H), 8.09 (tdd, *J* = 7.9, 5.1, 1.8 Hz, 1H), 7.87 – 7.78 (m, 4H), 7.77 – 7.70 (m, 2H), 7.62 (tdd, *J* = 9.1, 4.5, 3.2 Hz, 5H), 7.57 (d, *J* = 8.0 Hz, 1H), 6.71 (dd, *J* = 1.6, 0.9 Hz, 1H), 6.63 (dd, *J* = 8.0, 1.6 Hz, 1H), 3.75 – 3.62 (m, 2H), 2.92 –

2.82 (m, 2H), 1.72 – 1.46 (m, 4H), 1.37 – 1.07 (m, 22H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 206.6, 162.8, 151.9 (d, *J* = 19.1 Hz), 147.9, 145.4, 144.2, 138.8 (d, *J* = 10.3 Hz), 135.1 (d, *J* = 3.0 Hz), 134.1 (d, *J* = 9.6 Hz), 132.5 (d, *J* = 24.5 Hz), 130.5 (d, *J* = 12.6 Hz), 130.1, 128.1 (d, *J* = 3.5 Hz), 120.3, 118.6, 117.9 (d, *J* = 85.4 Hz), 117.3, 38.4, 30.6 (d, *J* = 15.2 Hz), 29.72, 29.70, 29.68, 29.63, 29.58, 29.56, 29.46, 29.32, 29.28, 24.8, 22.62, 22.59 (d, *J* = 13.6 Hz), 22.1, 22.0. HRMS (ESI$^+$) *m/z* 608.3666 ($C_{40}H_{51}PNO_2$ requires: 608.3657).

(16-(2,4-Dihydroxyphenyl)-16-oxohexadecyl)tri(naphthalen-1-yl)phosphonium bromide (4). Compound **6a** (53.6 mg, 0.13 mmol) and tris(naphthalen-1-yl)phosphane (67.0 mg, 0.16 mmol) were suspended in 2.0 mL of anhydrous DMF in a Kimax tube under inert atmosphere. The tube was screw-capped and the mixture was kept under stirring at 110 ºC for 5 days. The solvent was evaporated under high vacuum and the crude solid was purified by flash chromatography on silica gel (Hexane/EtOAc: 0→20%) leading to a mixture of **2** and tri(naphthalen-1-yl)phosphine oxide. The latter was removed by preparative TLC (EtOAc/MeOH: 8/2) and **4** was obtained as pale white solid (7 mg, 6.4%). HPLC > 95%. ^{1}H NMR (500 MHz, Methanol-d_4) δ 8.43 (dd, *J* = 8.5, 1.5 Hz, 3H), 8.20 – 8.11 (m, 6H), 7.97 (d, *J* = 8.6 Hz, 3H), 7.78 – 7.61 (m, 7H), 7.54 – 7.47 (m, 3H), 6.33 (ddd, *J* = 8.8, 2.4, 0.6 Hz, 1H), 6.20 (d, *J* = 2.4 Hz, 1H), 4.00 (br, 2H), 2.88 (t, *J* = 7.4 Hz, 2H), 1.68 (p, *J* = 7.4 Hz, 2H), 1.63 – 0.95 (m, 22H). HRMS (ESI$^+$) *m/z* 759.3981 ($C_{52}H_{56}O_3P$ requires 759.3967).

16-Bromohexadecanoic acid (5). A solution of 16-hydroxyhexadecanoic acid (1.0 g, 3.67 mmol) in acetic acid (4 mL) and HBr 48% aq (w/w) (4 mL) was stirred at 110 ºC for 41 h in a screw-capped Kimax tube. When cooled, a precipitate formed at the top of the solution. The precipitate was filtered, washed with water, and dried under high vacuum, obtaining 1.671 g of crude product. Recrystallization from hexane yielded **5** as white crystals (1.074 g, 87%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 3.34 (t, *J* = 6.9 Hz, 2H), 2.28 (t, *J* = 7.5 Hz, 2H), 1.79 (p, *J* = 7.2 Hz, 2H), 1.57 (p, *J* = 7.5 Hz, 2H), 1.40 – 1.12 (m, 22H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 179.9, 34.03, 34.02, 32.9, 29.62, 29.60, 29.58, 29.54, 29.44, 29.42, 29.2, 29.1, 28.8, 28.2, 24.7.

16-Bromo-1-(2,4-dihydroxyphenyl)hexadecan-1-one (6a). An oven-dried Kimax tube was charged with resorcinol (33 mg, 0.3 mmol) and 16-bromohexadecanoic acid **5** (104 mg, 0.3 mmol). The tube was flushed with argon and boron trifluoride diethyl etherate

(1.5 mL) was added at once. The tube was screw-capped and stirred at 110 ºC for 2 h. The cold reaction mixture was poured into crushed ice and the product was extracted with EtOAc (25 mL). The organic phase was washed with brine (5 mL), dried ($MgSO_4$), and evaporated to give a crude brown oil. Silica chromatography (10 g) eluting with hexane/EtOAc: 100/0→90/10 yielded the product as colorless glassy solid (58 mg, 45%). HPLC (UV) >90%. ^{1}H NMR (300 MHz, Chloroform-*d*) δ 12.81 (s, 1H), 7.59 (d, *J* = 8.9 Hz, 1H), 6.40–6.20 (m, 2H), 6.11 (brs, 1H), 3.34 (t, *J* = 7.0 Hz, 2H), 2.82 (t, *J* = 7.5 Hz, 2H), 1.78 (p, *J* = 7.1 Hz, 2H), 1.65 (p, *J* = 7.3 Hz, 2H), 1.45 – 1.06 (m, 20H). ^{13}C NMR (75 MHz, Chloroform-*d*) δ 205.6, 165.4, 162.7, 132.6, 114.0, 107.9, 103.7, 38.2, 34.3, 33.0, 29.76, 29.73, 29.67, 29.61, 29.57, 29.52, 28.9, 28.3, 25.1.

16-Bromo-1-(4-hydroxy-2-methylphenyl)hexadecan-1-one (6b). Same procedure as **6a** starting from *m*-cresol (30.6 mg, 0.28 mmol). Silica chromatography with hexane/EtOAc: 100/0 →30/1 yielded an impure product as colorless paraffin-like solid (75 mg). The product was purified further by silica chromatography using hexane 100% to remove the impurity ethyl 16-bromohexadecanoate. Off-white paraffin-like solid (15 mg, 13%). HPLC (UV) > 95%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 12.36 (s, 1H), 7.57 (d, *J* = 8.3 Hz, 1H), 6.72 (d, *J* = 1.5 Hz, 1H), 6.63 (dd, *J* = 8.3, 1.5 Hz, 1H), 3.34 (t, *J* = 6.9 Hz, 2H), 2.89 – 2.84 (m, 2H), 2.28 (s, 3H), 2.25 – 2.18 (m, 2H), 1.83 – 1.74 (m, 2H), 1.66 (p, *J* = 7.4 Hz, 2H), 1.59 – 1.50 (m, 2H), 1.39 – 1.17 (m, 18H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 206.5, 162.8, 147.9, 130.0, 120.2, 118.7, 117.3, 38.4, 34.6, 34.2, 33.0, 29.74, 29.73, 29.68, 29.61, 29.59, 29.58, 29.49, 29.41, 29.29, 28.91, 28.33, 25.14, 24.86.

(14-Hydroxytetradecyl)triphenylphosphonium bromide (9). Using the same procedure as above for compound **2**, a mixture of 14-bromotetradecan-1-ol **8**[11] (37.6 mg, 0.13 mmol) and Ph_3P (33.6 mg, 0.13 mmol) in dry acetonitrile (1 mL) was stirred at 80 ºC for 4 days under argon atmosphere. The solvent was removed under vacuum and the product was isolated by precipitation from MeOH/Et_2O. The precipitate was rinsed with Et_2O to yield **9** as colorless solid (44 mg, 61%). HPLC > 95%. ^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.85 – 7.69 (m, 9H), 7.64 (ddd, *J* = 8.5, 6.6, 3.5 Hz, 6H), 3.82 – 3.66 (m, 2H), 3.56 (t, *J* = 6.7 Hz, 2H), 1.61 – 1.43 (m, 6H), 1.31 – 1.07 (m, 18H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 135.1 (d, *J* = 3.0 Hz), 133.8 (d, *J* = 10.0 Hz), 130.6 (d, *J* = 12.5 Hz), 118.5 (d, *J* = 85.8 Hz), 62.9, 32.9, 30.5 (d, *J* = 15.6 Hz), 29.57, 29.53, 29.51, 29.48, 29.44, 29.3, 29.2, 25.8, 22.9 (d, *J* = 49.7 Hz), 22.7 (d, *J* = 4.6 Hz). HRMS (ESI$^+$) *m/z* 475.3127 ($C_{32}H_{44}OP$ requires: 475.3130).

Physicochemical properties. pK_a and logP were measured by potentiometry using the SIRIUS T3 apparatus. As compound **1** is poorly soluble in water, its pK_a was measured in H_2O/MeOH mixture and extrapolated to 0% co-solvent using the Yasuda-Shedlovsky procedure as implemented in the SIRIUS T3 Refine software. The thermodynamic solubility measurements were performed by a miniaturized shake-flask method and analyzed by UV-spectrophotometry.

Biology

Chemical compounds

For the biological assays, stock solutions of synthesized compounds in DMSO at 10 mM were prepared. Triton X-100 (TX100), carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP), m-chlorophenylhydrazone (CCCP), amphotericin B (AmB), 4',6-diamidino-2-phenylindole dilactate (DAPI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO). Miltefosine (MIL) was purchased from Zentaris GmbH (Frankfurt, Germany). L-glutamine and penicillin/streptomycin were obtained from Gibco. Cell-Titer Glo was purchased from Promega. JC-1, H_2DCFDA, bis-(1,3-dibutylbarbituric acid)trimethine oxonol [DiBAC4(3)] and Sytox Green were purchased from Invitrogen (Carlsbad, CA). All chemicals were of the highest quality available.

Leishmania cell lines and culture

Leishmania donovani (MHOM/ET/67/HU3) line was employed in the screening of synthesized compounds. *Leishmania infantum* LEM 3049 line[24] was employed as the reference line to perform the experiments related to mechanism of action, uptake and *in vivo* sensitivity. Three resistant lines obtained from clinical cases, namely *L. infantum* LEM 3323 (a line clinical resistant to Sb^{III})[24], *L. infantum* LEM 5159 (a line clinical resistant to MIL)[24] and *L. infantum* LEM 2126 (a line clinical resistant to MIL and paromomycin)[25] were used for sensitivity assays. All these lines were grown at 28 °C in RPMI 1640-modified medium (Invitrogen) supplemented with 10% hiFBS (Invitrogen), as described.[36]

Sensitivity analysis and *in vitro* infection of THP-1 derived macrophages with *Leishmania* lines

The sensitivity of *Leishmania* promastigote lines to the different compounds was determined after incubation for 72 h at 28 °C in presence of increasing concentrations of the compounds. The concentration of compound required to inhibit 50 % of parasite growth (EC_{50}) was calculated using the MTT colorimetric assay, as described previously.[37] For the intracellular *Leishmania* amastigotes' susceptibility to compounds, stationary-phase promastigotes were used to infect macrophage-differentiated THP-1 cells at a macrophage/parasite ratio of 1:10, as described previously.[37] After overnight infection at 35 ºC with 5% CO_2 in RPMI 1640 medium plus 5% hiFBS, extracellular parasites were removed by washing with serum-free medium. Infected macrophages were incubated with different concentrations of compounds in RPMI 1640 medium plus 10% hiFBS at 37 ºC and a 5% CO_2 atmosphere, for 96 h. Following incubation, samples were fixed for 20 min at 4 ºC with 2.5% paraformaldehyde in PBS buffer and then permeabilized with 0.1% TX100 in PBS for 10 min. Infection level (54.3 ± 5.6%; control) and mean number of intracellular parasites/cell (3.6 ± 0.7; control) were detected by nuclear staining with Prolong-Gold antifade agent with DAPI (Invitrogen). The percentage of infection and the mean number of amastigotes per infected macrophage were determined in 200 macrophages/well.

Human cell lines culture and determination of cellular toxicity

Human myelomonocytic cell line THP-1 was grown in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (hiFBS), 2 mM glutamate, 100 U/mL penicillin and 100 mg/mL streptomycin at 37 °C and 5% CO_2. 5×10^5 THP-1 cells per well in 24-well plates were differentiated to macrophages with 20 ng/mL of PMA treatment for 48 h followed by 24 h of culture in complete fresh medium.

MRC-5 cells, a SV-40 transformed human fetal lung fibroblast cell line, were maintained at 37 °C and 5% CO_2 in DMEM supplemented with 10% hiFBS, 100 U/mL penicillin and 100 μg/mL streptomycin.

The cellular toxicity of all compounds was determined using the colorimetric MTT-based assay,[38] as described for *Leishmania* promastigotes, except for the incubation temperature, which was 37 °C in this case.

ATP measurement in *Leishmania* parasites

ATP was measured using a CellTiter-Glo® luminescence assay as described[39] with modifications. Briefly, parasites (4×10^6/mL promastigotes) were incubated with 0.02, 0.1 and 1 μM compound **1** for different time-points (0, 30, 60, 120 min and 24 h) before being mixed with the same volume of CellTiter-Glo® (Promega) and incubated in the dark for 10 min following the manufacturer instructions. The bioluminescence was measured using an Infinite® F200 Luminescence System (Tecan Austria GmbH).

Measurement of mitochondrial membrane potential in promastigote forms of *Leishmania*

The mitochondrial membrane potential ($\Delta\Psi_m$) was measured using the JC-1 fluorescent marker as previously described.[39] JC-1 is a cationic fluorescent compound (green colour as monomer, FL1 emission at 530 nm after excitation at 490 nm) which exhibits potential-dependent accumulation in mitochondria, as indicated by a shift in its fluorescence emission from green to red (FL2 emission at 590 nm) due to the formation of red fluorescent aggregates. Depolarization of $\Delta\Psi_m$ is indicated by a reduced accumulation of the dye and a shift from red to green with a decrease in the red/green fluorescence ratio. A cell suspension (4×10^6 parasites per mL) was treated with 0.02, 0.1 and 1 μM of compound **1** for 30, 60, 120 min and 24 h, and then further incubated at 28 ºC with 5 μM JC-1 in HBS buffer for 10 min. Cellular fluorescence was quantified by calculating the ratio between FL2 and FL1 in a FACScan flow cytometer (Becton-Dickinson, San Jose, CA). The uncoupling agent FCCP (10 μM), was used as a control for mitochondrial depolarization.

Determination of plasma membrane integrity in *Leishmania* parasites

Cell membrane permeability was determined using the Sytox Green nucleic acid stain as described with modifications.[40] *L. infantum* promastigotes (2×10^6) were incubated in HBS buffer with 0.02, 0.1 and 1 μM compound **1** for 2 h. As a positive control we used parasites permeabilized with 1% TX100. After compound **1** treatment, parasites were incubated in the presence of 0.5 μM SYTOX Green for 30 min at 28 ºC. The parasites where then washed and analyzed by flow cytometry using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA). Fluorescence emission was quantified at 523 nm wavelength upon excitation at 490 nm by using Cell Quest software.

Determination of plasma membrane depolarization in *Leishmania* parasites

The membrane potential-sensitive probe $DiBAC_4(3)$ was used to measure potential changes as previously described previously.[38] Thus, parasites (1×10^7 promastigotes) were incubated without or with different concentrations of compound **1** (0.02, 0.1 and 1 μM) for 2 h in culture medium at 28 °C and then treated with 2 μM DiBAC4(3) for 10 min at 28 °C. Parasites treated with a 10 μM concentration of the depolarizing agent carbonyl cyanide m-chlorophenylhydrazone (CCCP), for 15 min were used as controls. DiBAC4(3) fluorescence was analyzed by flow cytometry using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA).

Measurement of ROS accumulation in *Leishmania* parasites

Intracellular ROS accumulation was measured using the fluorescent probe H_2DCFDA as described with some modifications.[28] Briefly, 1×10^7 *Leishmania* promastigotes were incubated in HBS buffer (21 mM HEPES, 0.7 mM Na_2HPO_4, 137 mM NaCl, 5 mM KCl, and 6 mM glucose, pH 7) containing 1 μM of compound **1** for 2 h at 28 ºC. As a positive control we used parasites incubated with 100 μM H_2O_2. After this treatment, parasites were washed and incubated in the presence of 0.1 mM H_2DCFDA for 30 min at 28 ºC. After incubation, the parasites were washed again with HBS buffer and analyzed by flow cytometry using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA). Fluorescence was measured at 517–527 nm wavelengths upon excitation at 490 nm by using Cell Quest software.

DNA fragmentation analysis

DNA fragmentation was analyzed by terminal deoxynucleotidyltransferase (TdT)-mediated dUTP end labeling (TUNEL) using the Roche in situ cell death detection kit as described previously.[41] Parasites (10^7 promastigotes) were incubated with 0.02, 0.1, 1, and 5 μM of compound 1 for 4 h at 28°C in culture medium, fixed with 4% formaldehyde in PBS for 15 min at room temperature, and permeabilized with 0.1% Triton X-100 for 2 min at 4°C. Then, the parasites were labeled with TUNEL reaction mixture following the manufacturer's instructions. Fluorescence was measured by flow cytometry, in a FACScan flow cytometer (Becton-Dickinson, San Jose, CA). Unfixed parasites were

labeled with 0.4 μg/ml PI for 5 min at 4°C and measured by flow cytometry to detect necrotic cells.

Uptake of compound 4 and effect of temperature and energy

Promastigotes (1×10^8 cells/mL) were treated with 20 μM of compound **4** for 1, 5, 10, 30 and 60 min in culture medium at 28 ºC. Afterwards, parasites were washed with the same medium followed by phosphate-buffered saline (PBS; 1.2 mM KH_2PO_4, 8.1 mM NaH_2PO_4, 130 mM NaCl and 2.6 mM KCl, pH 7), and re-suspended in 1% TX-100. Compound **4** accumulation was determined fluorimetrically by recording an emission spectrum in the range 510 ± 20 nm upon excitation at 340 nm an Infinite® F200 Luminescence System (Tecan Austria GmbH).

For energy-depletion studies, parasites were pre-incubated for 10 min with 5 mM 2-deoxy-D-glucose and 20 mM NaN_3 at 28 °C in HBS buffer without glucose. To analyze the effect of the temperature in the uptake, we treated the parasites with compound **4** and determined the uptake at 28 ºC (control) and 4 ºC.

Statistical analysis

Statistical comparisons between groups were performed using the Student's t-test. Differences were considered significant at a level of $p< 0.05$.

Pharmacokinetics

A pharmacokinetic study was performed with male BALB/c mice to determine the oral bioavailability of compound **1**. In the snapshot experiment, the compound was suspended in 8% DMSO in water and a single dosage of 20 mg/kg was administered orally (n = 3 mice). Serum samples were obtained by facial vein puncture at 0, 1, 3, 6, 24, 48 and 72 h post administration. Serum samples were deproteinized by adding 1.5 mL of acetonitrile, mixed in a vortex and centrifuged at 1370 × *g* for 10 min. Supernatant was filtered through 0.45 μm mesh (Millex® HV PVDF, Merck Millipore) and assayed by modular HPLC (Jasco, Japan). Liver and spleen samples were weighed and mixed with diluted acetic acid. After homogenization, the samples were subjected to three freezing/thawing cycles to extract the compound. Acetonitrile (0.75 mL) was added, mixed and centrifuged at 1370 × *g* for 10 min. Supernatant was filtered and assayed by HPLC as above. The flow rate was 1.5 mL/min of a mobile phase containing a mixture 80:20 of acetonitrile:purified

water containing 0.05% orthophosphoric acid. The stationary phase was a BDS Hypersil® C18 column (Thermo Scientific, USA) 5 μm, 200 × 4.6 mm. The injection volume was 100 μL and the detection wavelength was 275 nm. Apparent half-lives were calculated based on a first order elimination rate.

Microsomal stability

Microsomal stability of compound **1** towards metabolism by cytochrome P450 (Phase-I metabolism) and Uridine Glucuronosyl-Transferase (UGT) (Phase-II metabolism) was studied in presence of NADPH and UDPGA, respectively. Incubation media (0.6 mL, final volume) containing human liver microsomes (HLM) (Gentest; Corning) (0.66 mg prot./mL), mouse (CD-1) liver microsomes (Sigma-Aldrich) (0.66 mg prot./mL) or human liver S9 fraction (Sigma-Aldrich) (0.83 mg prot./mL) in 80 mM potassium phosphate buffer (pH 7.4) were added with compound **1** (5–10 μM) or diclofenac (20–40 μM) and NADPH (1 mM), and incubated in a water bath at 37 ºC for 2 h. On the other hand, incubation media (0.6 mL, final volume) containing human or mouse liver microsomes (0.66 mg prot./mL) or human liver S9 mix fraction (0.83 mg prot./mL) in 80 mM potassium phosphate buffer (pH 7.4), and 4 mM $MgCl_2$ were sonicated for 3 min with tubes in ice bath and added with compound **1** (10–20 μM), UDPGA (2 mM), and incubated at 37 ºC for 2 h. Aliquots (100 μL) from incubation media were withdrawn at 0, 15, 30, 60 and 120 min and added to 100 μL of acetonitrile, vortexed and centrifuged at 10000 rpm. An aliquot of supernatant (20 μL) was analyzed by RP-HPLC (Agilent 1200 apparatus with a 1200 diode array detector (DAD) using a reversed phase 3.9×150 mm, 4 μm, Nova-pak C18 column (Waters, Milford, MA, USA) under the following chromatographic conditions: eluent A: 50 mM ammonium phosphate buffer (pH 3) and eluent B: 20 % A in acetonitrile. A linear gradient was used from 0 to 100% B in 8 min, and then 100% B for 10 min. Under these conditions **1** eluted at t = 11.7 min. Compound **1** was detected at 215 and 275 nm and diclofenac at 280 nm. Assays were carried out at least in duplicate and the maximum amount of DMSO or CH_3CN was 0.25% and 0.5%, respectively.

In vivo assays

Animals and ethics statement. BALB/c mice (six-week-old, female) from Charles River Breeding Laboratories were housed and fed under pathogen-free conditions in the Animal Facility Service of the Instituto de Parasitología y Biomedicina “López-Neyra”, CSIC.

The use of laboratory rodents was carried out in strict accordance to all mandatory guidelines (EU directives, including the Revised Directive 2010/63/EU on the Protection of Animals used for Scientific Purposes that came into force on 1 January 2013 and the declaration of Helsinki in its latest version) and was approved by the ethical committee of the Instituto de Parasitología y Biomedicina "López-Neyra" – CSIC (file CEA-03-11-2018). Pharmacological studies (experimental design and housing conditions) were approved by the Committee of Animal Experimentation (Universidad Complutense de Madrid) and regional authorities (Community of Madrid) (Ref. PROEX 169/15). Experiments were carried out at the animal house ES280790001164 following the 3Rs principles. Animal handling and sampling were performed by trained and officially qualified personnel.

Analysis of *in vivo* infection

Animals (5 mice/group) were injected intraperitoneally with 5×10^7 stationary-phase *L. infantum* promastigotes resuspended in PBS (following the technical recommendation of our Animal Facility in order to establish the most homogeneous infections rate). Two weeks after infection, the parasite burden with this *Leishmania* line reached the maximum as routinely determined in our Animal Facility under their experimental conditions. Two weeks after infection, animals were treated orally for 4 days with 1.5 mg/kg of compound **1** suspended in 5% (w/w) γ-cyclodextrin (Ashland Industries Europe, Cavamax® W8, Switzerland) in distilled water. Two days after the end of the treatment, spleen and liver were removed for parasite quantification using the limiting dilution technique as described previously.[42, 43] Briefly, target tissues were homogenised in 5 mL of RPMI 1640 medium supplemented with 20 % hiFBS, 100 U/mL penicillin and 100 µg/mL streptomycin using a tissue grinder. After centrifuging at 100 × *g* for 10 min at 4 °C, the supernatant was collected and centrifuged at 2000 × *g* for 20 min at 4 °C. The pellet was resuspended in 5 mL of RPMI 1640 medium and then dispensed in 96-well plates using progressive 1:10 dilutions. Parasite burden was determined using light microscopy to examine the dilution which yielded promastigotes after 7 days of incubation at 28 °C.

ASSOCIATED CONTENT

Supporting Information Available.

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Activity of compounds **10**–**40** in promastigote and intracellular amastigote forms of *L. donovani* HU3 and cytotoxicity against MRC-5 or THP-1 cells (Table S1). Plasma concentration of compound **1** in BALB/c mice after oral administration (Table S2). Microsomal stability of compound **1** toward metabolism by cytochrome P450 and Uridine Glucuronosyl-Transferase (Table S3). ^{1}H and ^{13}C NMR spectra of compounds **1**–**4** and **9**.

Molecular formula strings and activity data (CSV)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

AmB, amphotericin B; BSF trypanosome, bloodstream form trypanosome; CCCP. carbonyl cyanide m-chlorophenylhydrazone; DAPI, 4',6-diamidino-2-phenylindole dilactate; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; hiFBS, heat-inactivated fetal bovine serum; MIL, miltefosine; MoA, mechanism of action; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide; PMA, phorbol 12-myristate 13-acetate; RFU, relative fluorescence units; ROS, reactive oxygen species; SI, selectivity index; TPP, triphenylphosphonium; TAO, trypanosome alternative oxidase; UGT, uridine glucuronosyl-transferase

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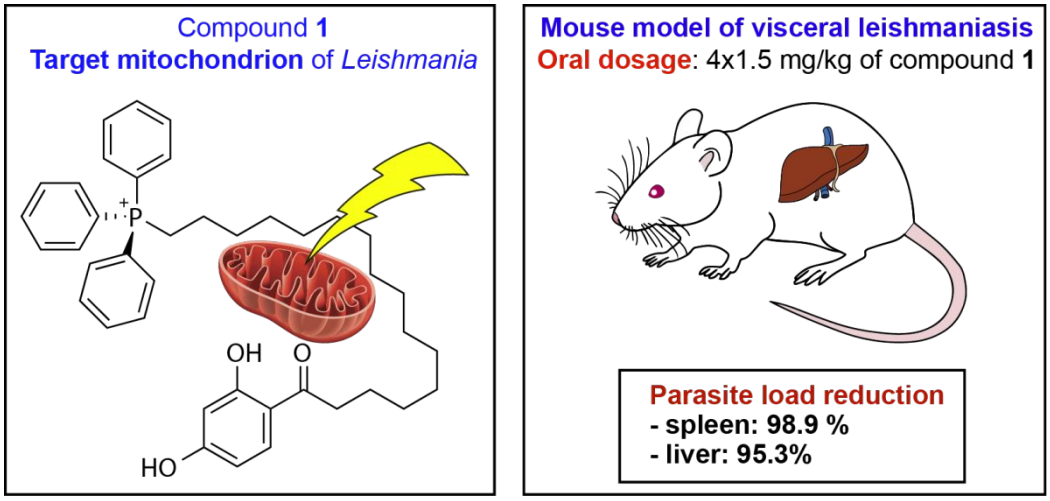
TOC Graphic


Compound 1
Target mitochondrion of Leishmania
P
OH
O
HO
Mouse model of visceral leishmaniasis
Oral dosage: 4x1.5 mg/kg of compound 1
Parasite load reduction
- spleen: 98.9 %
- liver: 95.3%