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In vivo and in vitro anti-leishmanial activities of 4-nitro-*N*-pyrimidin- and *N*-pyrazin-2-ylbenzenesulfonamides, and N^2-(4-nitrophenyl)-N^1-propylglycinamide

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ABSTRACT

A series of compounds containing the nitrobenzene and sulfonamido moieties were synthesized and their leishmanicidal effect was assessed in vitro against *Leishmania infantum* promastigotes. Among the compounds evaluated, the *p*-nitrobenzenesulfonamides **4Aa** and **4Ba**, and the *p*-nitroaniline **5** showed significant activity with a good selectivity index. In a Balb/c mice model of *L. Infantum*, administration of compounds **4Aa**, **4Ba** or **5** (5 mg/kg/day for 10 days, injected ip route) led to a clear-cut parasite burden reduction (ca. 99%). In an attempt to elucidate their mechanism of action, the DNA interaction of **4Aa** and **5** was investigated by means of viscosity studies, thermal denaturation and nuclease activity assay. Both compounds showed nuclease activity in the presence of copper salt. The results suggest that compounds **4Aa**, **4Ba** and **5** represent possible candidates for drug development in the therapeutic control of leishmaniasis.

1. Introduction

Protozoan parasitic diseases have plagued mankind for centuries and continue to cause significant public health problems. Leishmaniasis comprises a group of diseases with distinct clinical manifestations caused by different species of the protozoan parasite *Leishmania*. This type of infection is considered within the most relevant group of neglected tropical diseases and targeted by the WHO and other health organizations for prevention, control and eradication.[1,2] It is estimated that human leishmaniasis is distributed worldwide with a prevalence of 12 million individuals in 88 countries and 350 million individuals at risk of infection.[3,4] In addition, over the last decade *Leishmania*-HIV co-infections are becoming a major health problem in affected areas.[5,6]

Current treatment of leishmaniasis is based on chemotherapy with some attempts at immunotherapy. Unfortunately, there is no vaccine against *Leishmania* infection and vector control is difficult. Therefore chemotherapy is the main approach to control this worldwide spread disease.[7–11] Pentavalent antimonials have been the classic treatment of leishmaniasis. Pentamidine, amphotericin B and miltefosine have been introduced as second-line therapy. However, the resistance to antimonials has become a severe problem and treatment with second-line drugs is generally expensive and frequently complicated by the occurrence of toxic side effects.[12–14] Consequently, the development of novel, efficient and safe chemotherapeutic agents with leishmanicidal activity is imperative.

In this context, sulfonamides have long been the subject of pharmaceutical interest as a result of their potent biological activities.[15,16] Recently, it has been established that sulfonamides in the presence of Cu(II) and reducing agents have strong nuclease activity.[17] Moreover, sulfonamide derivatives exhibiting anti-leishmanial activity have been described.[18] Thus, a series of *N*-quinolin-8-yl-arylsulfonamides have been reported as potent in vitro leishmanicidal compounds, being the quinolinsulfonamide **1** (Fig. 1) the most active compound.[19] On the other hand it has been shown that nitro aromatic derivatives possess substantial activity against *Leishmania*. In those studies compounds **2** and **3** were identified as new anti-leishmanial compounds.[20–22]

Based on the anti-leishmanial activity of nitrobenzene derivatives and aryl sulfonamides, we synthesized a series of com-

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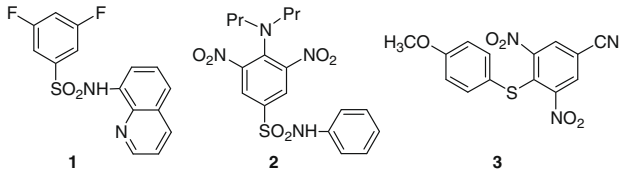


Figure 1. Sulfonamides and nitrobenzene derivatives with leishmanicidal activity.

pounds that have both nitrobenzene and sulfonamide entities and we evaluated their leishmanicidal effect against *Leishmania infantum* promastigotes (Fig. 2). Compounds exhibiting a good selectivity index were also tested in vivo in an acute murine model of leishmaniasis. Studies to elucidate the mechanism of action were also carried out and the interaction with DNA was investigated by viscosity studies, thermal denaturation and nuclease activity assays.

Herein we report on the evaluation of compounds **4–7** as potential antiparasitics against *L. infantum*. The results demonstrate that the benzenesulfonamides **4Aa** and **4Ba**, and the *p*-nitroaniline derivative **5** are promising candidates to be considered as lead compounds for new leishmanicidal drugs, since they are effective against promastigotes of *L. infantum* at concentrations in the micromolar range and display excellent in vivo efficacies in a mouse model of leishmaniosis, with no toxicity against mammalian cells. Compounds **4Aa** and **5** showed nuclease activity in the presence of copper salt, therefore it is not possible to discard DNA cleavage for their mechanism of action.

2. Results and discussion

2.1. Leishmanicidal activity

The compounds synthesized to investigate the anti-leishmanial activity were sulfonamides **4A–F**, sulfadiazine, *p*-nitroaniline **5**, guanidines **6a–f** and imidazolidinones **7a,b** (Fig. 2).

Sulfonamides **4** were easily obtained by reaction of the amine with the corresponding sulfonyl chloride according to the literature[23] (Scheme 1). The synthesis of *p*-nitroaniline **5**, guanidines **6** and imidazolidinones **7** was carried out according to a procedure previously described.[24]

The leishmanicidal activity of sulfadiazine and compounds **4**, **5**, **6** and **7** was evaluated in vitro against cultured promastigotes of *L. infantum* (MCAN/ES/92/BCN 83), according to a modification of a previously reported procedure.[25] Whereas guanidines **6** and imidazolidinones **7** had no effect on the growth of *L infantum* promastigotes at concentrations as high as 100 μg/mL, pyrimidines **4Aa,b,k**, pyrazine **4Ba**, pyridine **4Bd**, thiazoles **4Ca,b,d–f**, isoxazole 4Da, pyrazol**e 4Dd**, indazole **4E**, indanes **4Fa–c** and aniline **5** were found to be active compounds. The *p*-nitrobenzenesulfonamides **4Aa** (92.7% growth inhibition, GI) **4Ba** (90.8% GI), **4Da** (85.6% GI), **4E** (88% GI) and **4Fa** (91.9% GI), and the *p*-nitroaniline **5** (85% GI) were the most active compounds. In the case of compounds **4Aa**, **4Ba**, **4Da**, **4E** and **5**, the leishmanicidal activity was associated with a lack of toxicity to murine macrophages (IC_{50} >256 μM) (Table 1).

As shown in Table 1, the anti-leishmanial activity of sulfonamides **4A** follows the order **4Aa** > **4Ab** > **4Ae** ≈ **4Ai**, being the *p*-nitro derivative **4Aa** the most active compound. The removal of the

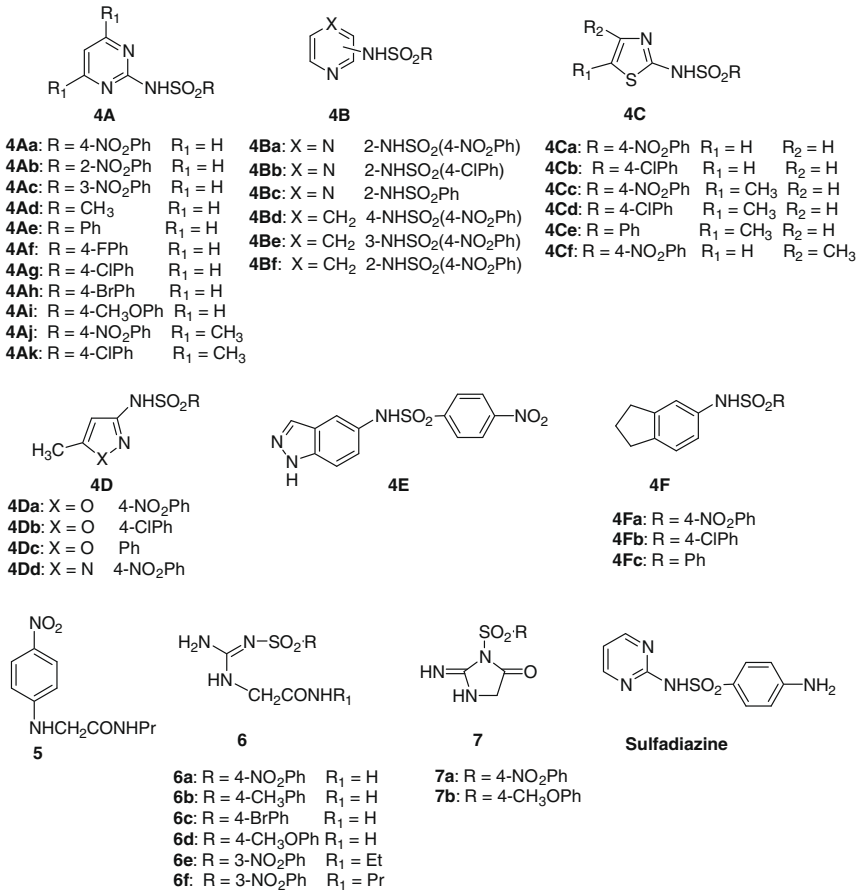
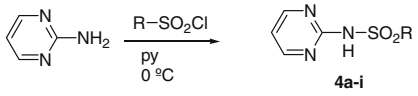


Figure 2. Structures of the series of compounds evaluated against *L. infantum*.



Scheme 1. Synthesis of sulfonamides **4.**

Table 1
In vitro activities of compounds **4**, **5** and sulfadiazine against *L. infantum* promastigotes and J774 macrophages[a,b]

Compound	Growth inhibition (%)	IC_{50} (μM)	
		L. infantum	J774
4Aa	92.7	100 ± 2.6	>256
4Ab	40.6	>256	ND
4Ac	—	—	—
4Ad	—	—	—
4Ae	20.3	>256	ND
4Af	—	—	—
4Ag	—	—	—
4Ah	—	—	—
4Ai	29.6	>256	ND
4Aj	33.3	>256	ND
4Ak	71.5	224 ± 32.2	>256
4Ba	90.8	66.2 ± 3.4	>256
4Bb	25.3	>256	ND
4Bc	37.0	>256	ND
4Bd	46.3	>256	ND
4Be	35.7	>256	ND
4Bf	39.3	>256	ND
4Ca	58.6	>256	ND
4Cb	65.0	>256	ND
4Cc	36.7	>256	ND
4Cd	74.5	>256	ND
4Ce	69.0	>256	ND
4Cf	55.6	>256	ND
4Da	85.6	126 ± 0.7	>256
4Db	15.7	>256	ND
4Dc	17.9	>256	ND
4Dd	71.5	164 ± 10.7	>256
4E	88.2	14.5 ± 0.2	>256
4Fa	91.9	<1	61 ± 5.6
4Fb	49.9	>256	ND
4Fc	45.3	>256	ND
Sulfadiazine	—	—	—
5	85.0	156 ± 26	>256

[a] ND, not determined.
[b] —, No activity detected.

electron withdrawing nitro group, as in sulfonamide **4Ae**, decreases the potency against the parasites and the replacement of the nitro group on the benzene ring of compound **4Aa** by an electron-donating methoxy group as in compound **4Ai**, causes a diminution in the antiparasitic activity. In addition, sulfonamides **4Af** and **4Ah** with electron-withdrawing substituents in *para* position were also ineffective. The same pattern is observed in the pyridazine (**4Ba–c**), isoxazole (**4Da–c**) and indane (**4Fa–c**) series. Therefore, the electronic effect of the substituents on the benzene ring seems to be not too relevant for the leishmanicidal activity and our findings indicate that the *p*-nitrobenzene moiety is essential for the antiparasitic activity.

In an attempt to explain the lack of activity of the other two *p*-nitrophenyl derivatives **6a** and **7a**, we were suspect of a higher hydrophilic character of **6a** and **7** to enter the parasite in comparison to **4Aa**, **4Ba** and **5**. We used the software ACD/Labs to estimate their log *P*. The log *P* values of **6a** (0.10) and **7a** (−0.34) were significantly lower than **4Aa** (1.04), **4Ba** (1.20) and **5** (1.54).

It is noteworthy that the substitution of the NO_2 group of compound **4Aa** by NH_2 (antibacterial sulfadiazine) resulted in a significant loss of activity. This finding suggests that the observed activity is not probably due to the reduced form of compound **4Aa** ($NO_2 \rightarrow NH_2$) and the mode of action of **4Aa** on *L. infantum* by the classical route of inhibiting the novo folate synthesis could be discarded. Probably, sulfonamides **4** and *p*-nitroaniline **5** enter the parasite and undergo redox cycling producing reactive oxygen species (ROS) that can peroxidatively damage membrane lipids, proteins or DNA.[12] In fact, several nitroaromatic compounds have been shown that increase ROS generation in *Leishmania* parasites.[21]

Sulfonamides **4Aa, 4Ba**, and *p*-nitroaniline **5**, the most active compounds in the in vitro anti-promastigote assay with a good therapeutic index, were then tested in vivo in an acute murine model of visceral leishmaniosis. Compounds **4Aa, 4Ba** and **5** were assayed at concentrations of 5 mg/kg/dose administered by the intraperitoneal route daily up to a total of 10 doses using a modification of a method previously described.[26] The assayed compounds displayed high levels of activity, with significant suppression of parasite burden in spleen and liver compared to the parasite burden in the tissues of the controls (Table 2). No apparent signs of toxicity, such as gross weight loss or hair loss, were observed in any of the animals at the end of the assay, strongly suggesting that **4Aa, 4Ba** and **5** are well tolerated by the infected mice.

2.2. DNA binding properties

In an attempt to gain insight into the anti-leishmanial mechanism of action of compounds **4Aa** and **5**, the mode and propensity for binding to calf thymus DNA (CT-DNA) was investigated by viscosity studies, thermal denaturation and nuclease activity assay.

Viscosity measurements were carried out on calf thymus DNA by varying the concentration of sulfonamide **4Aa** or *p*-nitroaniline **5**. Hydrodynamic measurements that are sensitive to length changes are regarded as the least ambiguous and the most critical tests of binding in solution. A classical intercalation model results in the lengthening of the DNA helix as the base pairs are separated to accommodate the binding ligand, leading to an increase of DNA viscosity. By contrast, compounds that bind exclusively in DNA grooves by partial and/or non-classical intercalation under the same conditions typically cause a less pronounced (positive or negative) or no change in DNA solution viscosity.[27] The results obtained with compounds **4Aa** or **5** reveal that both of them have no effect on the relative viscosity of CT-DNA at 25 °C or at 37 °C. These findings indicate that the interaction does not involve a classic intercalation.

Thermal behaviour of DNA in the presence of some compounds can give insight into their conformational changes when temperature is raised and information about the interaction strength of the complexes with DNA. The double-stranded DNA tends to dissociate gradually to single strands when increasing the solution temperature thus generating a hyperchromic effect on the absorption spectra of DNA bases (λ_{max} = 260 nm). In order to identify this transition process, the melting temperature (T_m), which is defined as the temperature where the half of the double strand plasmid is

Table 2
In vivo anti-leishmanial effect of compounds **4Aa**, **4Ba** and **5**

Compound	Mean ± SD[b]% suppression[a]	
	Spleen	Liver
4Aa	99.82 ± 0.11[c]	97.58 ± 4.03[c]
5	99.89 ± 0.10[c]	97.95 ± 3.88[c]
4Ba	99.44 ± 0.93[c]	99.28 ± 0.92[c]

[a] Reductions of parasite burdens in spleens and livers of treated mice in relation to those in the control (untreated) groups.
[b] The standard deviation (SD) was calculated by comparing individual data for the treated animals with the average value for the control group.
[c] P <0.0005 compared with controls.

transformed into a single strand one, is a valuable parameter.[28] Intercalation of natural or synthesized organics and metallointercalators generally results in a considerable increase in the melting point temperature (T_m).[29] The results indicate that both compounds **4Aa** and **5** do not produce significant changes in the T_m value (T_m control = 53.8 °C, T_m **4Aa** = 54.2 °C, T_m **5** = 54.0 °C) and therefore no stabilization of DNA takes place in the presence of compounds **4Aa** and **5**.

The activity of compounds **4Aa** and **5** as chemical nucleases was studied using supercoiled pUC18 DNA in cacodylate buffer (pH 6.0). Figure 3 shows that sulfonamide **4Aa** and *p*-nitroaniline **5** did not present ability to damage DNA plasmid at the assayed concentrations. Further assays were carried out in order to evaluate the ability to cleave pUC18 plasmid DNA by copper(II)–ligand (1:1) mixtures. The nuclease activity of the mixtures ranging from 15 to 45 μM was examined and compared with that of the cupric chloride in the presence of ascorbate (50-fold excess relative copper(II) concentration) (Fig. 4). At concentrations of 15 μM or 30 μM, the Cu(II)–ligand (1:1) mixtures were not able to induce DNA cleavage, whereas at the concentration of 45 μM a complete degradation of supercoiled form to yield circular and linear forms was induced (lanes 10 and 14). This activity was higher than that of copper(II) salt at the same conditions (compare lanes 10 and 14 with lane 6). Taking into consideration that the ligands do not exhibit nuclease activity alone, it is evident that Cu(II) do contribute to the DNA cleavage process. The results indicate that **4Aa** and **5** do not interact nor cleave the DNA by themselves but they do so in the presence of copper salt. This behaviour is probably due to a redox process in the presence of the metal transition. The activation of a Fenton-type or a Haber–Weiss reactions produces the generation of oxygen active species (hydroxyl, superoxide, singlet oxygen-like species and hydrogen peroxide) which then cleave the DNA.[30] Further studies will make possible to identify the reactive oxygen species that mediate the DNA strand scission by monitoring the quenching of DNA cleavage in the presence of ROS scavengers in solution.

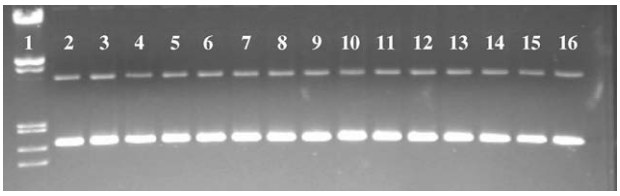


Figure 3. Agarose gel electrophoresis of pUC18 plasmid DNA treated with compounds **4Aa** and **5**. Incubation time 60 min (37 °C). (1) λDNA/EcoR1 + HindIII Marker; (2) control; (3) **4Aa** 6 μM; (4) **4Aa** 9 μM; (5) **4Aa** 12 μM; (6) **4Aa** 15 μM; (7) **4Aa** 30 μM; (8) **4Aa** 45 μM; (9) **4Aa** 60 μM; (10) **5** 6 μM; (11) **5** 9 μM; (12) **5** 12 μM; (13) **5** 15 μM; (14) **5** 30 μM; (15) **5** 45 μM; (16) **5** 60 μM.

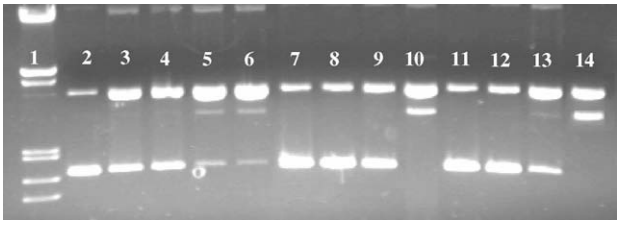


Figure 4. Agarose gel electrophoresis of pUC18 plasmid DNA treated with the mixture of copper chloride and the ligands and 50-fold-excess of ascorbate. Incubation time 60 min (37 °C). (1) λDNA/EcoR1 + HindIII Marker; (2) control; (3) pUC18 + ascorbate; (4) $CuCl_2$ 15 μM; (5) $CuCl_2$ 30 μM; (6) $CuCl_2$ 45 μM; (7) **4Aa** 45 μM; (8) $CuCl_2$ + **4Aa** 15 μM; (9) $CuCl_2$ + **4Aa** 30 μM; (10) $CuCl_2$ + **4Aa** 45 μM; (11) **5** 45 μM; (12) $CuCl_2$ + **5** 15 μM; (13) $CuCl_2$ + **5** 30 μM; (14) $CuCl_2$ + **5** 45 μM.

3. Conclusion

In summary, sulfonamides **4Aa, 4Ba** and the *p*-nitroaniline **5** constitute new antiparasitic agents with high selectivity indices versus mammalian cells and emerge as candidates for further evaluation against kinetoplastid parasites. Since the synthesis of these compounds is straightforward, a wide variety of analogues can be prepared to optimize in vitro and in vivo activities. In addition, given the economic realities of chemotherapy for leishmaniasis, the inexpensive large-scale synthesis of appropriate candidates would allow their future development if in vivo activity can be achieved. Synthesis and pharmacological evaluation of related derivatives to determine structural features for the anti-leishmanial activity are currently in progress.

4. Experimental section

4.1. Chemistry

4.1.1. General

All reagents were purchased from Aldrich and used without purification. All experiments were made under nitrogen atmosphere. Melting points were determined with a Kofler hot-stage apparatus and are uncorrected. Column chromatography was performed using silica gel (Merck 60, 70–230 mesh). ^1H and ^{13}C NMR spectra were recorded on a Bruker AC-300 instrument. Chemical shifts (δ values) and coupling constants (J values) are given in ppm and hertz, respectively. HRMS were obtained using a VG Autospec TRIO 1000 instrument. The ionization mode used in mass spectra was electron impact (EI) or fast atom bombardment (FAB). Sulfadiazine was obtained from Sigma Chemical Co. Compounds **4Aa**, **4Ad**, **4Ah, 4Ai**, **5**, **6a–d** and **7a,b** were synthesized as previously described.[24]

4.1.2. General procedure for the synthesis of sulfonamides 4[23]

To an ice-cooled solution of 2-aminopyrimidine (1.9 g, 20 mmol) in pyridine (8 mL) was slowly added the corresponding sulfonyl chloride (30 mmol) in pyridine (6 mL). The mixture was stirred at 0 °C for 2 h and allowed to reach room temperature. Water was added (100 mL) and the solid was collected and recrystallized from $MeOH/CH_2Cl_2$.

4.1.2.1. 2-Nitro-*N*-pyrimidin-2-ylbenzenesulfonamide (4Ab). Yield: 93%; mp: 137–139 °C; ^1H NMR (300 MHz, DMSO-d_6): δ = 6.98 (t, J = 4.8 Hz, 1H), 7.24 (td, J = 8.4, J = 1.3, 1H), 7.71 (td, J = 8.4, J = 1.5, 1H), 8.05 (dd, J = 8.4, J = 1.5, 1H), 8.17 (dd, J = 8.4, J = 1.3, 1H), 8.51 (d, J = 4.8, 2H), 10.11 (s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 114.6 (CH), 123.0 (CH), 123.6 (CH), 125.8 (CH), 130.9 (C), 135.0 (CH), 139.7 (C), 158.5 (CH), 159.3 (C); HRMS-FAB$^+$: m/z [M+H] calcd for $C_{10}H_9N_4O_4S$: 281.0344, found: 281.0339.

4.1.2.2. 3-Nitro-*N*-pyrimidin-2-ylbenzenesulfonamide (4Ac) Yield: 50%; mp: 217–220 °C (lit[31] 217–219 °C); ^1H NMR (300 MHz, DMSO-d_6): δ = 7.06 (t, J = 4.9, 1H), 7.89 (t, J = 8.1, 1H), 8.40 (d, J = 8.1, 1H), 8.46 (d, J = 8.1, 1H), 8.52 (d, J = 4.9, 2H), 8.70 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 114.9 (CH), 122.7 (CH), 127.3 (CH), 131.1 (CH), 133.8 (CH), 143.4 (C), 147.9 (C), 157.4 (C), 158.5 (CH); HRMS (FAB$^+$): m/z [M+H] calcd for $C_{10}H_9N_4O_4S$: 281.0344, found: 281.0356.

4.1.2.3. *N*-Pyrimidin-2-ylbenzenesulfonamide (4Ae). Yield: 97%; mp: 227–229 °C (lit[32] 229–230 °C); ^1H NMR (300 MHz, DMSO-d_6): δ = 7.03 (t, J = 4.9, 1H), 7.56 (m, 3H), 7.96 (dd, J = 7.9, J = 1.3, 2H), 8.47 (d, J = 4.9, 2H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 116.0 (CH), 127.8 (CH), 129.2 (CH), 133.2 (CH), 140.0 (C), 157.4 (C), 158.6 (CH).

4.1.2.4. 4-Fluoro-*N*-pyrimidin-2-yl-benzenesulfonamide (4Af) Yield: 80%; mp: 183–186 °C (lit[33] 184.5–185 °C); ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.05 (t, J = 4.9, 1H), 7.41 (t, J = 9.0, 2H), 8.03 (dd, J = 9.0, J = 5.0, 2H), 8.50 (d, J = 4.9, 2H), 11.0 (s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 116.1 (CH), 116.5 (CH), 131.0 (CH), 136.9 (C), 157.4 (C), 158.7 (CH), 162.5 (C).

4.1.2.5. 4-Chloro-*N*-pyrimidin-2-yl-benzenesulfonamide (4Ag) Yield: 80%; mp: 199–202 °C (lit[34] 203–204 °C); ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.06 (t, J = 4.9, 1H), 7.63 (d, J = 8.1, 2H), 7.98 (d, J = 8.1, 2H), 8.51 (d, J = 4.9, 2H), 12.03 (s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 116.1 (CH), 129.4 (CH), 129.8 (CH), 138.1 (C), 139.7 (C), 157.0 (C), 158.7 (CH).

4.1.2.6. *N*-(4,6-Dimethyl-pyrimidin-2-yl)-4-nitro-benzenesulfonamide (4Aj). Yield: 65%; mp: 218–220 °C (lit.[37] 215 °C); ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.25 (s, 6H), 6.72 (s, 1H), 8.18 (d, J = 8.8, 2H), 8.35 (d, J = 8.8, 2H).

4.1.2.7. *N*-(4,6-Dimethyl-pyrimidin-2-yl)-4-chloro-benzenesulfonamide (4Ak). Yield: 54%; mp: 185–187 °C (lit.[38] 181–182 °C); ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.25 (s, 6H), 6.75 (s, 1H), 7.62 (d, J = 8.5, 2H), 7.96 (d, J = 8.5, 2H), 12.14 (s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 22.9 (CH_3), 113.1 (CH), 128.8 (CH), 130.2 (C), 130.4 (CH), 137.4 (C), 140.5 (C), 156.2 (C).

4.1.2.8. 4-Nitro-*N*-pyrazin-2-yl-benzenesulfonamide (4Ba) Yield: 86%; mp: 233–237 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 8.2 (m, 4H), 8.40 (m, 3H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 124.9 (CH), 129.2 (CH), 133.7 (CH), 139.5 (CH), 142.1 (CH), 145.8 (C), 148.1 (C), 150.3 (C); HRMS-EI$^+$: m/z calcd for $C_{10}H_9N_4O_4S$: 281.0344, found: 281.0351.

4.1.2.9. 4-Chloro-*N*-pyrazin-2-yl-benzenesulfonamide (4Bb) Yield: 40%; mp: 190–193 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.67 (d, J = 8.5, 2H), 7.95 (d, J = 8.5, 2H); 8.22 (dd, J = 2.8, J = 1.5, 1H), 8.22 (d, J = 1.5, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 129.5 (CH), 129.7 (CH), 135.5 (CH), 138.4 (C), 139.2 (CH), 139.3 (C), 142.3 (CH), 148.4 (C), HRMS-EI$^+$: m/z calcd for $C_{10}H_9N_3O_2SCl$: 270.0104, found: 270.0107.

4.1.2.10. *N*-Pyrazin-2-yl-benzenesulfonamide (4Bc). Yield: 70%; mp: 204–208 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.60 (m, 3H), 7.93 (d, J = 8, 2H); 8.22 (m, 2H), 8.35 (d, J = 1.3, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 127.4 (CH), 129.6 (CH), 133.6 (CH), 135.2 (CH), 139.2 (CH), 140.2 (C), 142.4 (CH), 148.4 (C); HRMS-FAB$^+$: m/z calcd for $C_{10}H_{10}N_3O_2S$: 236.0493, found: 236.0495.

4.1.2.11. 4-Nitro-*N*-pyridin-4-yl-benzenesulfonamide (4Bd) Yield: 40%; mp: 290–294 °C (lit.[39] 293–295 °C); ^{1}H NMR (300 MHz, DMSO-d_6): δ = 6.97 (d, J = 7.53, 2H), 8.02 (d, J = 7.53, 2H), 8.04 (d, J = 8.86, 2H), 8.31 (d, J = 8.86, 2H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 115.4 (CH), 124.5 (CH), 127.8 (CH), 139.4 (C), 139.5 (CH), 149.1 (C), 150.0 (C).

4.1.2.12. 4-Nitro-*N*-pyridin-3-yl-benzenesulfonamide (4Be) Yield: 88%; mp: 240–243 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.33 (dd, J = 8.2, J = 4.8, 1H), 7.51 (ddd, J = 8.2, J = 2.6, J = 1.5, 1H), 8.02 (d, J = 8.85, 2H), 8.31 (m, 2H), 8.37 (d, J = 8.85, 2H), 10.9 (s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 124.5 (CH), 125.2 (CH), 128.6 (CH), 128.7 (CH), 134.0 (C), 142.6 (CH), 144.7 (C), 146.3 (CH), 150.3 (C); HRMS-EI$^+$: m/z calcd for $C_{11}H_9N_3O_4S$: 279.0313, found: 279.0299.

4.1.2.13. 4-Nitro-*N*-pyridin-2-yl-benzenesulfonamide (4Bf) Yield: 63%; mp: 185–186 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 6.86 (td, J = 7.1, J = 0.94, 1H), 7.27 (d, J = 8.8, 1H), 7.81 (td J = 9, J = 1.8, 1H), 7.94 (dd, J = 7.1, J = 1.8, 1H), 8.09 (d, J = 8.8, 2H), 8.34 (d, J = 8.8, 2H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 114.4 (CH), 115.5 (CH), 124.6 (CH), 128.6 (CH), 128.0 (CH), 140.3 (C), 142.8 (CH), 149.0 (C), 149.4 (CH), 154.6 (C); HRMS-EI$^+$: m/z calcd for $C_{11}H_9N_3O_4S$: 279.0313, found: 279.0314.

4.1.2.14. 4-Nitro-*N*-thiazol-2-yl-benzenesulfonamide (4Ca) Yield: 90%; mp: 263–265 °C (lit[40] 260–261 °C); ^{1}H NMR (300 MHz, DMSO-d_6): δ = 6.95 (d, J = 3.3, 1H), 7.30 (d, J = 3.3, 1H), 8.11 (d, J = 8.5, 2H), 8.40 (d, J = 8.5, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 109.4 (CH), 124.8 (CH), 125.2 (CH), 127.6 (CH), 148.1 (C), 149.6 (C), 169.7 (C).

4.1.2.15. 4-Chloro-*N*-thiazol-2-yl-benzenesulfonamide (4Cb) Yield: 90%; mp: 198–200 °C (lit.[34] 201–202 °C); ^{1}H NMR (300 MHz, DMSO-d_6): δ = 6.80 (d, J = 3.3, 1H); 7.25 (d, J = 3.3, 1H); 7.6 (d, J = 8.5, 2H); 8.80 (d, J = 8.5, 2H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 109.0 (CH); 125.0 (CH); 128.0 (CH); 129.4 (CH); 137.1 (C); 141.6 (C); 169.4 (C).

4.1.2.16. 4-Nitro-*N*-5-methyl-thiazol-2-yl-benzenesulfonamide (4Cc). Yield: 88%; mp: 235–240 °C (lit.[41] 255 °C); ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.17 (s, 3H), 7.05 (s, 1H), 8.03 (d, J = 8.5, 2H), 8.35 (d, J = 8.5, 2H).

4.1.2.17. 4-Chloro-*N*-5-methyl-thiazol-2-yl-benzenesulfonamide (4Cd). Yield: 50%; mp: 102–104 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.06 (s, 3H); 6.33 (s, 1H); 7.58 (d, J = 8.5, 2H); 7.75 (d, J = 8.5, 2H); HRMS-EI$^+$: m/z calcd for $C_{10}H_9ClN_2O_2S_2$: 287.9793, found: 287.9794.

4.1.2.18. *N*-5-Methyl-thiazol-2-yl-benzenesulfonamide (4Ce) Yield: 40%; mp: 149.1–152 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.15 (s, 3H), 6.46 (s, 1H), 7.61 (m, 3H), 7.85 (d, J = 8.3, 2H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 13.7 (CH_3); 102.6 (CH); 116.4 (C); 126.1 (CH); 129.2 (CH); 132.2 (CH); 135.0 (C); 165.8 (C).

4.1.2.19. 4-Nitro-*N*-4-methyl-thiazol-2-yl-benzenesulfonamide (4Cf). Yield: 70%; mp: 189.5–193.2 °C (lit.[42] 197–9 °C). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.10 (s, 3H), 6.45 (s, 1H), 8.05 (d, J = 8.5, 2H); 8.37 (d, J = 8.5, 2H).

4.1.2.20. *N*-4-Methyl-thiazol-2-yl-benzenesulfonamide (4Cg) Yield: 57%; mp: 164–166 °C (lit.[43] 161–162 °C). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.17 (s, 3H); 6.97 (s, 1H); 7.55 (m, 3H); 7.77 (d, J = 8.1, 2H).

4.1.2.21. *N*-(5-Methyl-isoxazol-3-yl)-4-nitro-benzenesulfonamide (4Da). Yield: 90%; mp: 190–193 °C (lit.[44] 194–195 °C); ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.29 (s, 3H), 6.16 (s, 1H), 8.10 (d, J = 9.2, 2H), 8.42 (d, J = 9.2, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 12.4 (CH_3), 95.8 (CH), 125.2 (CH), 128.8 (CH), 144.9 (C), 149.5 (C), 157.4 (C), 171.0 (C).

4.1.2.22. *N*-(5-Methyl-isoxazol-3-yl)-4-chlorobenzenesulfonamide (4Db). Yield: 60%; mp: 158.5–159.9 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.29 (s, 3H); 6.14 (s, 1H); 7.70 (d, J = 8.8, 2H); 7.85 (d, J = 8.8, 2H), 11.5 (br s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 12.4 (CH_3), 95.8 (CH), 129.0 (CH), 129.9 (CH), 138.6 (C), 138.7 (C), 149.5 (C), 157.6 (C), 171.0 (C); HRMS-EI$^+$: m/z calcd for $C_{10}H_9N_2O_3SCl$: 272.0022, found: 282.0019.

4.1.2.23. *N*-(5-Methyl-isoxazol-3-yl)-benzenesulfonamide (4Dc) Yield: 90%; mp: 105–108 °C (lit.[45] 112.5 °C); ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.29 (s, 3H); 6.14 (s, 1H); 7.62 (m, 3H); 7.87 (d,

J = 8.2, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 12.4 (CH_3), 95.7 (CH), 127.0 (CH), 129.8 (CH), 133.8 (CH), 139.7 (C), 157.6 (C), 171.0 (C).

4.1.2.24. *N*-(5-Methyl-1*H*-pyrazol-3-yl)-4-nitro-benzenesulfonamide (4Dd). Yield: 95%; mp: 167–171 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.94 (s, 3H), 5.15 (s, 1H), 8.13 (d, J = 8.8, 2H), 8.40 (d, J = 8.8, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 14.2 (CH_3), 89.4 (CH), 125.3 (CH), 129.1 (CH), 142.3 (C), 146.0 (C), 152.6 (C), 152.8 (C). HRMS-EI$^+$: m/z calcd for $C_{10}H_{10}N_4O_4S$: 282.0422, found: 282.0423.

4.1.2.25. *N*-(1*H*-Indazol-5-yl)-4-nitro-benzenesulfonamide (4E) Yield: 73%; mp: 234–236 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.08 (d, J = 8.8, 1H), 7.44 (m, 2H), 7.93 (d, J = 8.7, 2H), 8.00 (s, 1H), 8.31 (d, J = 8.7, 2H), 10.5 (br s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 111.2 (CH), 114.1 (CH), 122.9 (CH), 123.2 (C), 124.9 (CH), 128.7 (CH), 129.6 (C), 133.9 (CH), 138.2 (C), 145.1 (C), 150.0 (C); HRMS-EI$^+$: m/z calcd for $C_{13}H_{10}N_4O_4S$: 318.0422, found: 318.0429.

4.1.2.26. *N*-Indan-5-yl-4-chloro-benzenesulfonamide (4Fb) Yield: 84%; mp: 121–123 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.80 (m, 2H), 2.69 (m, 4H), 6.78 (dd, J = 7.9, J = 1.3, 1H), 6.90 (s, 1H), 7.00 (d, J = 7.9, 1H), 7.54 (d, J = 8.2, 2H), 7.68 (d, J = 8.2, 2H), 10.1 (br s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 25.4 (CH_2), 32.0 (CH_2), 32.6 (CH_2), 117.4 (CH), 119.5 (CH), 125.0 (CH), 129.0 (CH), 129.7 (CH), 135.7 (C), 137.9 (C), 138.8 (C), 140.4 (C), 145.1 (C); HRMS-EI$^+$: m/z calcd for $C_{15}H_{14}ClNO_2S$: 307.0433, found: 307.0439.

4.1.2.27. *N*-Indan-5-yl-benzenesulfonamide (4Fc). Yield: 91%; mp: 150–154 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.92 (m, 2H), 2.72 (m, 4H), 6.84 (dd, J = 8.1, J = 2.1, 1H), 6.94 (s, 1H), 7.02 (d, J = 8.1, 1H), 7.53 (m, 3H), 7.72 (d, J = 7.9, 2H), 10.1 (br s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 25.4 (CH_2), 31.9 (CH_2), 32.6 (CH_2), 117.1 (CH), 118.9 (CH), 124.9 (CH), 127.0 (CH), 129.5 (CH), 134.0 (CH), 136.0 (C), 139.9 (C), 140.0 (C), 145.4 (C); HRMS-EI$^+$: m/z calcd for $C_{15}H_{15}NO_2S$: 273.0823, found: 273.0826.

4.1.3. Synthesis of guanidines (6e) and (6f)

Prepared as described for analogues **6a–d**,[24] starting from compound **8**.

4.1.3.1. 2-[(2-{[(3-Nitrophenyl)sulfonyl]imino}pyrimidin-1(2*H*)-yl]acetamide (8). To a stirred suspension of sulfonamide **4c** (1 g, 3.6 mmol) in dimethylformamide, DMF (12 mL), diisopropylethylamine, DIPEA (0.65 mL, 3.6 mmol) was added dropwise under nitrogen. After 40 min iodoacetamide (0.66 g, 3.6 mmol) was added. The reaction mixture was stirred at room temperature for 16 h and then poured onto water (100 mL). The resulting solid was collected and dried to give compound **8**. Yield: 1.1 g (90%); mp: 240 °C (decomp); ^{1}H NMR (300 MHz, DMSO-d_6): δ = 4.79 (s, 2H, CH_2), 6.94 (dd, J = 6.6, J = 4.5, 1H), 7.46 (s, 1H, NH), 7.78 (t, J = 8.1, 1H), 7.86 (s, 1H, NH), 8.20 (ddd, J = 8.1, J = 1.7, J = 0.9, 1H), 8.35 (ddd, J = 8.1, J = 1.7, J = 0.9, 1H), 8.42 (dd, J = 6.6, J = 2.2, 1H), 8.56 (t, J = 1.7, 1H), 8.64 (dd, J = 4.5, J = 2.2, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 55.1 (CH_2), 109.1 (CH), 122.0 (CH), 126.2 (CH), 130.6 (CH), 133.4 (CH), 145.5 (C), 147.6 (C), 152.4 (CH), 154.9 (C), 164.9 (CH), 167.1 (C=O).

4.1.3.2. 2-[2-(3-Nitrobenzenesulfonyl)guanidino]-*N*-ethylacetamide (6e). To a solution of **8** (0.54 g, 1.63 mmol) in methanol (20 mL), ethylamine 2 M in methanol (5 mL) was added. The reaction mixture was heated at reflux for 6 h and then concentrated under reduced pressure. The remaining oil was purified by column chromatography. Eluent AcOEt/MeOH (9:1). Yield: 0.19 g (38%); mp: 170–172 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 0.97 (t, J = 7.3, 3H), 3.04 (quintet, J = 7.3, 2H), 3.72 (d, J = 5.2, 2H), 6.96 (s, 1H, NH), 7.08 (s, 1H, NH), 7.81 (t, J = 8.1, 1H), 7.95 (t, J = 5.2, 1H, NH), 8.16 (dd, J = 7.7, J = 1.3, 1H), 8.37 (ddd, J = 7.7, J = 2.2, J = 1.3, 1H), 8.45 (t, J = 2.2, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 14.4 (CH_3), 33.2 (CH_2), 43.2 (CH_2), 120.2 (CH), 125.8 (CH), 130.6 (CH), 131.7 (CH), 145.6 (C), 147.1 (C), 156.6 (C=N), 167.6 (C=O); HRMS-EI$^+$: m/z calcd for $C_{11}H_{15}N_5O_5S$: 329.0793, found: 329.0788.

4.1.3.3. 2-[2-(3-Nitrobenzenesulfonyl)guanidino]-*N*-propylacetamide (6f). A solution of **8** (0.54 g, 1.63 mmol) in propylamine (5 mL) was heated at reflux for 6 h. The excess of amine was removed under reduced pressure and the residue was purified by column chromatography. Eluent AcOEt. Yield: 0.34 g (61%); mp: 108–110 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 0.80 (t, J = 7.3, 3H), 1.35 (sextet, J = 7.3, 2H), 2.96 (q, J = 6.5, 2H), 3.72 (d, J = 5.2, 2H), 6.96 (s, 1H, NH), 7.07 (s, 1H, NH), 7.80 (t, J = 8.10, 1H), 7.95 (t, J = 5.2, 1H, NH), 8.16 (d, J = 8.1, 1H), 8.37 (d, J = 8.1, 1H), 8.45 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 11.6 (CH_3), 22.6 (CH_2), 40.6 (CH_2), 43.6 (CH_2), 120.7 (CH), 126.3 (CH), 131.1 (CH), 132.2 (CH), 146.0 (C), 147.9 (C), 157.1 (C=N), 168.2 (C=O); HRMS-EI$^+$: m/z calcd for $C_{12}H_{17}N_5O_5S$: 343.0950, found: 343.0952.

4.2. Leishmanicidal assays

4.2.1. In vitro leishmanicidal assay

4.2.1.1. Parasite. An autochthonous isolate of *L. infantum* (MCAN/ES/92/BCN 83) obtained from an asymptomatic dog from the Prior rat region (Catalunya, Spain) and given by Professor Montserrat Portús was used. Promastigotes were cultured in Schneider's Insect Medium (Sigma, St. Louis, MO) at 26 °C supplemented with 20% heat-inactivated foetal bovine serum (FBS) (Sigma, St. Louis, MO) and 100 U/mL of penicillin plus 100 μg/mL of streptomycin (Sigma, St. Louis, MO) in 25 mL culture flasks.

4.2.1.2. Promastigote assay. The assay was performed using a modification of a previous method.[25] Promastigotes (2.5×10^5parasites/well) were cultured in 96-well plastic plates. Compounds were dissolved in dimethylsulfoxide (DMSO). Different dilutions of the compounds (a first screening using 1, 10 and 100 μg/mL, and then 252, 126, 63, 31.5, 15.7, 7.8, 3.9, 1.9 μM for active compounds) up to 200 μL final volume were added. After 48 h at 26 °C, 20 μL of 2.5 mM resazurin solution was added and the fluorescence intensity (535 nm-excitation wavelength- and 590 nm-emission wavelength-) was determined with a fluorometer Infinite 200 (Tecan i-Control) to calculate growth inhibition (%). The solution of resazurin was prepared at 2.5 mM in phosphate buffered solution (PBS), pH 7.4, and filtered through 0.22 μm prior use. All tests were carried out in triplicate. Resazurin sodium salt was obtained from Sigma–Aldrich (St. Louis, MO) and stored at 4 °C protected from light.

4.2.2. Cytotoxicity assay

4.2.2.1. Cells. J774 murine macrophages were grown in RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated FBS (30 min at 56 °C), penicillin G (100 U/mL) and streptomycin (100 μg/mL). For the experiments, cells in the pre-confluence phase were harvested with trypsin. Cell cultures were maintained at 37 °C in a humidified environment with 5% CO_2.

4.2.2.2. Cytotoxicity assay on macrophages. Cell viability was evaluated using a modification of a method previously described.[35] J774 macrophages were seeded (5×10^4 cells/well) in 96-well flat-bottom microplates with 200 μL of RPMI 1640 medium. The cells were allowed to adhere for 24 h at 37 °C in 5% CO_2, then the medium was replaced by different concentrations of the test compounds in 200 μL of medium and exposed to for another 24 h. Growth controls were also included. To evaluate cell viability, 20 μL of a 2.5 mM resazurin solution were added and the plates were returned to the

incubator for another 3 h. Reduction of resazurin was determined by measurement of the fluorescence intensity (535 nm-excitation wavelength- and 590 nm-emission wavelength-). Background was subtracted. Each concentration was assayed three times. Medium and controls were used in each test as blanks.

4.2.3. In vivo leishmanicidal assay

4.2.3.1. Mice. Balb/c mice of 6–8 weeks of age were purchased from Harlan Ibérica S.A. (Barcelona, Spain) and allocated in the Animal House Unit of the Complutense University under controlled food, light/darkness cycles and temperature conditions. All procedures for animal manipulations were approved by the Complutense University Institutional Animal Care and Use Committee following Spanish law.

4.2.3.2. Preparation of *Leishmania infantum* parasites for experimental infection. *L. infantum* amastigotes (MCAN/ES/92/BCN 83) harvested from spleens of infected hamsters were cultured in NNN medium added with penicillin (200 IU) and streptomycin (2 mg/mL) for 2 days up to transformation into promastigotes. Thereafter they were transferred to C-199 medium supplemented with 1% 10 mM adenin in 50 mM Hepes, 0.25% hemin in 50% triethanolamine, 0.348 g/L sodium bicarbonate, 25 mM Hepes, 20% heat-inactivated foetal bovine serum (FBS, Sigma), 100 μg/mL penicillin and 100 μg/mL streptomycin, at pH 7.2. Under these conditions the maximum number of metacyclic forms as determined by its resistance to complement lysis is achieved by day 7th. After 7 days the primary culture promastigotes were harvested by centrifugation at 2000*g* for 15 min, washed with phosphate buffer saline (PBS) at pH 7.2 and finally resuspended in PBS. Promastigotes were counted using the Neubauer haemocytometer and the final suspension adjusted to provide 10^7 promastigotes per inoculum. Groups of 6 animals each were infected with 10^7 promastigotes per animal, given by the intracardiac (IC) route following anaesthesia with sodium pentobarbital.

4.2.3.3. Protocol. Mice were randomly assigned to three groups. One group was kept as untreated control. Treatment started on the 55 day post-infection and lasted for 10 continuous days. Animals were dosed once daily and the compounds were administered at 5 mg/kg given by the intraperitoneal route in a 0.1 mL final volume of physiological saline solution. Five days later, the mice were sacrificed and the parasitic burden was evaluated.

4.2.3.4. Estimation of parasite burden. The liver and spleen from each animal were aseptically removed, weighted and afterwards samples of each were homogenised in cold PBS-glucose-EDTA solution using a steel stainless tissue grinder. Cell debris was eliminated by passage trough a glass wool column. The suspension obtained was centrifuged at 2000*g* for 15 min at 4 °C. Thereafter the supernatants were discarded and the pellets resuspended in C-199 medium supplemented with 1% adenin, 0.2% hemin, sodium bicarbonate, 25 mM Hepes, antibiotics and 20% FBS. 200 mL of the suspension were transferred to each of the 96-well microtiter plate containing NNN medium added with antibiotics. Parasite burden were estimated by the limit dilution assay according to Hill et al. and Titus et al.[36]

4.3. DNA interactions

4.3.1. Viscosity measurements

Viscosity experiments were carried out with a semimicro Ubbelodhe viscosimeter immersed in a Julabo ME16G thermostatted water-bath maintained to 25.0 ± 0.1 °C or to 37.0 ± 0.1 °C. Solutions of the compounds in [DMF/cacodylate buffer (pH 6) 2:10] were added to a calf thymus DNA, CT-DNA (50 μM) solution in cacodylate buffer. Final concentrations ranged from 1 to 30 μM. The flow times were measured with a digital stopwatch. Time readings were recorded by triplicate. Data were analyzed as $(\eta/\eta_0)^{1/3}$ versus the ratio of the concentration of complex and DNA, where η is the viscosity of DNA in the presence of complex and η_0 is the viscosity of DNA alone. Viscosity values were calculated from the observed flow time of a DNA-containing solution corrected from the flow time of buffer alone (t_0), $\eta = t - t_0$.

4.3.2. Thermal denaturation experiments

DNA-melting experiments were carried out by monitoring the absorbance (260 nm) of CT-DNA (100 μM NP) at different temperatures in the absence or presence of the compounds at 4:1 DNA/complex ratio. Measurements were performed with an Agilent 8453 UV–vis spectrophotometer equipped with a Peltier temperature controlled sample cell and driver (Agilent 89090A). The solution containing the compound and CT-DNA in phosphate buffer (1 mM phosphate, 2 mM NaCl, pH 7.2) was continuously stirred and heated at 1 °C min^{-1} rate of temperature increase. The investigated interval of temperature ranged from 20 to 90 °C. The melting point was obtained with the first derivative using the Savitsky-Golay algorithm.

4.3.3. Nuclease activity: pUC18 DNA cleavage

Reactions of DNA cleavage produced by the compounds were undertaken by mixing 13 μL of 0.1 M cacodylate buffer (pH 6.0), 1 μL of pUC18 (0.25 μg/μl), 6 μL of a solution of the tested compound at increasing concentrations to obtain final concentrations between 6 μM and 60 μM. To assay nuclease activity in the presence of a copper salt, the samples contained 7 μL of 0.1 M cacodylate buffer (pH 6.0), 1 μL of pUC18 (0.25 μg/μL), 6 μL of a mixture $CuCl_2$ + compound solution at increasing concentrations to obtain final concentrations between 15 μM and 45 μM and 6 μL of reducing agent (ascorbate) in cacodylate buffer. The mixtures were allowed to stand for 1 h at 37 °C. After that, 3 μL of a loading buffer solution consisting of 0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol were added. Then the solution was subjected to electrophoresis on a 0.8% agarose gel in 0.5 × TBE buffer (0.045 M tris, 0.045 M boric acid and 1 mM EDTA) containing 2 μL/100 mL of a solution of ethidium bromide (10 mg/mL) at 80 V for about 2 h. The gel was photographed on a capturing Gel Printer plus TDI.

4.4. Statistics

The concentration of the test compounds that produced a 50% reduction in infected cells (IC_{50}) was determined from least-squares linear regression of growth rate. Mann–Whitney test was used to determine the statistical significance of the values obtained. Parasite suppression was determined by comparing each experimental value of the parasite burden with the mean value for the controls. Parasite burdens were analyzed by Student's *t*-test.

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