**Effective anti-Leishmanial activity of minimalist squaramide-based compounds**

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**Abstract**

In order to evaluate the *in vitro* leishmanicidal activity of N,N'-Squaramides derivatives, compounds that feature both hydrogen bond donor and acceptor groups and are capable of multiple interactions with complementary sites, against *Leishmania infantum*, *L. braziliensis* and *L. donovani* a series of 18compounds was prepared and assayed on extracellular and intracellular parasite forms. Infectivity and cytotoxicity tests were performed on J774.2 macrophage cells using meglumine antimoniate (Glucantime) as the reference drug. Changes in metabolite excretion by 1H-NMR and the ultrastructural alterations occurring in the parasites treated using transmission electron microscopy (TEM), was analyzed. Compounds **1, 7, 11, 14** and **17** were the more active and less toxic. Infection rates showed that the order of effectiveness was **17 > 11 > 14 > 7** for both *L. infantum* and *L. braziliensis* and in the same way, the compound **1** for *L. donovani*. All these compounds have altered the typical structure of the promastigotes, glycosomes and mitochondria. These severe modifications by the compounds are the ultimate reasons for the alterations observed in the excretion products. The Squaramide **17** was clearly the most efficient of all compounds. The data appear to confirm that the severe modifications generated in organelles such as glycosomes or mitochondria by the compounds are the ultimate reasons for the alterations observed in the excretion products of all species. The activity, stability, low cost of starting materials, and straightforward synthesis make amino squaramides appropriate molecules for the development of an affordable anti-leishmanial agent.

**Keywords:** Squaramides, *Leishmania infantum*, *L. braziliensis*, *L. donovani*, metabolite excretion, ultrastructural alterations.

1. **Introduction**

A total of 98 countries on 5 continents have reported endemic leishmaniasis transmission (WHO, 2015; Alvar et al. 2012). Leishmaniasis cause five main clinical manifestations: visceral leishmaniasis (VL) or kala-azar, cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), diffuse cutaneous leishmaniasis (DCL), and post-kala-azar dermal leishmaniasis (PKDL) (Fernandes et al., 2014).There are between 0.2 and 0.4 million of new VL and between 0.7 and 1.3 million of new CL cases each year (WHO, 2015). VL is considered the most severe form of the disease. Two specific species are responsible for VL around the world: *L*. *donovani* and *L*. *infantum*. CL is caused by different species of *Leishmania:* *L*. *mexicana*, *L*. *amazonensis*, *L*. *braziliensis*, or *L*. *panamensis* in the Americas and *L*. *tropica* and *L*. *major* in other countries (Fernandes et al., 2014). Drugs used for traditional treatment of these diseases are pentavalent antimonials, paromomycin, amphotericin-B and miltefosine. High toxicity and increasing resistance to the current chemotherapeutic regimens have further complicated the situation (Suryawanshi et al., 2013).

The chemotherapeutic investigation remains still very active, given the fact that, up to this time, there are not effective drugs that would completely cure these diseases. Many chemical and natural compounds have been studied as potential leishmanicidal drugs (Gupta et al., 2010; Alakurtti et al., 2010; Bhattacharya et al., 2013).Our research group has been working on this matter for many years, obtaining some promising results (Marin et al., 2013; Sánchez-Moreno et al., 2012; Ramirez-Macias et al., 2012).We have explored the potential of squaramide-based compounds as therapeutic agents: a series of oligomeric cyclosquaramides as kinase inhibitors with antitumor activity (Villalonga et al., 2012),antimalarials (Kumar et al., 2012) and antichagasic activity (Olmo et al., 2014).

Squaramides are going to become even more widespread in the near future, particularly in the fields pharmaceutical research and chemical biology (Prohens et al., 2001).In this regard, here we have explored the anti-leishmania properties of a selected group of minimalist mono- and bis-squaramide-based compounds (acidic, basic, neutral and amphoteric). It must be emphasized that the design of squaramide-based drugs is hampered by solubility limitations of squaramides both in water and in common organic solvents. The poor solubility observed is due to enhanced head-to-tail aggregation capabilities involving secondary squaramides. This propensity to aggregate can be counteracted by introducing donor atoms at the side chains (Martinez et al., 2012).Alternatively, the presence of charged groups also facilitates the solubility in aqueous solution (**Figure 1**) (López et al., 2012).

The squaramides depicted in **Figure 2** have reasonable druggability profiles in terms of Lipinski's rule of five (Rotger et al., 2006), a structural simplicity and affordable cost desirable for large productions. The anti-leishmania activity of squaramide compounds have been evaluated on promastigote and amastigote forms of *L. donovani, L. infantum* (VL etiological agents)and *L. braziliensis* (CL etiological agents).

1. **Material and methods**
	1. **Chemistry**

All reagents and solvents of commercial origin were used without further purification. Melting points were determined on a Dr. Tottoli (Büchi) apparatus and are uncorrected. Nuclear magnetic resonance (1H NMR and 13C NMR) spectra were recorded on Bruker Avance FT-NMR 300 MHz (1H), 75 MHz (13C) spectrometer. Chemical shifts are reported in ppm units and are referenced to the residual deuterated solvent resonance at 7.26/77.16 ppm for chloroform or 2.50/39.52 for dimethyl sulfoxide. Electrospray high-resolution mass spectra (ESI-HRMS) were recorded in positive ion monitoring mode on a Micromass Autospec3000 spectrometer provided with an electrospray module. RP-HPLC analysis and purity check were performed on a Gilson modular system equipped with a 321 pump and a UV-visible diode array detector, using a X-Bridge (150 × 4.6 mm, 5 µ) C18 column with the use of H2O-MeCN (10 – 90) in buffered solutions at pH=4 or 9.

Target squaramide compounds **1**–**18** were prepared by reported methods as depicted in **Scheme 1**. Briefly, compounds **1**–**9** and **14**–**17** were prepared from diethyl squarate by sequential condensation with two different amines or with a diamine and an amine, followed by a quaternization step with excess methyl iodide when necessary (Lipinski et al., 2001; Prohens et al., 2001; Martinez et al., 2012).

Derivatives **10**–**13** were prepared from squaric acid and the corresponding anilines under microwave irradiation (Lopez et al., 2013).Macrocyclic squaramide **18** was prepared in one step by a effective macrocyclization reaction of the diamine with diethylsquarate (Rotger et al., 2006).

*3-(benzylamino)-4-((1-benzylpiperidin-4-yl)amino)cyclobut-3-ene-1,2-dione* **(1)**.Yield 75%; mp 240°C (dec). 1H NMR (300 MHz, DMSO-d6) *δ*: 7.65 (br, 2H), 7.31 (m, 10H), 4.68 (d, *J* = 6.0 Hz, 2H), 3.74 (br, 1H), 3.73 (br, 2H), 2.70 (br, 2H), 2.03 (m, 2H), 1.81 (m, 2H), 1.45 (br, 2H). 13C NMR (75 MHz, DMSO-d6) *δ*: 188.0, 187.9, 172.8, 144.5, 143.9, 134.5, 134.3, 133.8, 133.3, 131.1, 132.5, 67.6, 56.8, 56.1, 52.4, 38.6. ESI-HRMS calcd for C22H25N3O2 M + H+ 376.2025, found 376.2023 M + H+.

*3-(butylamino)-4-((3-(dibutylamino)propyl)amino)cyclobut-3-ene-1,2-dione* **(2)**. Yield 80%; mp 116–118°C. 1H NMR (300 MHz, CDCl3) *δ*: 7.87 (br, 1H), 7.50 (br, 1H), 3.65 (m, 4 H), 2.52 (t, *J* =6.8 Hz, 2H), 3.39 (t, *J* = 7.11 Hz), 1.78 (m 2H), 1.62 (m, 2H), 1.34 (m, 14H), 0.92 (m, 9H). 13C NMR (75 MHz, CDCl3) *δ*:182.5, 182.4, 168.2, 168.1, 53.9, 51.5, 44.6, 43.5, 33.4, 29.2, 28.4, 20.9, 19.8, 14.3, 13.9. ESI-HRMS calcd for C19H35N3O2 M + H+ 338.2808, found 338.2795 M + H+.

*3-(butylamino)-4-((2-(dimethylamino)ethyl)amino)cyclobut-3-ene-1,2-dione* **(3)**. Yield 84%; mp 224°C (dec). 1H NMR (300 MHz, DMSO-d6) *δ*: 7.58 (br, 1H), 7.43 (br, 1H), 3.70 (d, *J* = 5.3 Hz, 2H), 3.61 (m, 2H), 2.49 (t, *J* = 5.9 Hz, 2H), 2.27 (s, 6H), 1.56 (m, 2H), 1.41 (m, 2H), 0.99 (t, *J* = 7.3 Hz, 3H). 13C NMR (DMSO-d6) *δ*: 182.4, 167.7, 167.5, 59.2, 45.0, 42.8, 41.0, 32.8, 19.0, 13.5. ESI-HRMS calcd for C12 H24N3O2Na M + Na+ 262.1531, found 262.1540 M + Na+.

*3-(hexadecylamino)-4-((2-(dimethylamino)ethyl)amino)cyclobut-3-ene-1,2-dione* **(4)**. Yield 65%; mp 171–173 °C. 1H NMR (300 MHz, CDCl3) *δ*: 7.46 (br, 1H), 7.31 (br, 1H), 3.6–3.58 (br, 2H), 3.48–3.46 (br, 2H), 2.38 (m, 2H), 2.16 (s, 6H), 1.49 (br, 2H), 1.23 (br, 26H), 0.84 (m, 3H). 13C NMR (CDCl3, 75 MHz): 182.6, 182.3, 168.2, 168.1, 59.8, 45.6, 44. 9, 42.4, 32.1, 31.4, 29.9, 29.5, 26.8, 22.8, 14.2. ESI-HRMS calcd. for C24H46N3O2 M + H+ 408.3590, found 408.3583 M + H+.

*2-((2-butylamino)-3,4-dioxocyclobut-1-en-1-yl)amino-N,N,N,-trimethylethan-1-aminium iodide* (**5**).Yield71%; mp 209–210°C; 1H NMR (300 MHz, DMSO-*d*6) *δ*: 7.68 (br s, NH), 7.55 (br s, NH), 4.03 (d, *J*=6.0 Hz, 2H), 3.61 (t, *J*=6.7 Hz, 4H), 3.22 (s, 9H), 1.58 (m, 2H), 1.41 (m, 2H), 0.99 (t, *J*=7.3 Hz, 3H) ppm. ESI-HRMS calcd. for C13H24N3O2: 254.1869. Found 254.1857 M – I+.

*2-((2-benzylamino)-3,4-dioxocyclobut-1-en-1-yl)amino-N,N,N,-trimethylethan-1-aminium iodide* (**6**) Yield 76%; mp 256–257°C; 1H NMR (300 MHz, DMSO-*d*6) *δ*: 8.05 (s, NH), 7.43 (m, 5H), 4.81 (br s, 2H), 4.05 (m, 2H), 3.61 (t, *J*=6.6 Hz, 2H), 3.2 (s, 9H) ppm; Anal. calcd for C16N3O2H22I: C, 46.28; H, 5.34; N, 10.12. Found: C, 45.28; H, 5.23; N, 9.83.

*2-((2-hexadecylamino)-3,4-dioxocyclobut-1-en-1-yl)amino-N,N,N,-trimethylethan-1-aminium iodide* (**7**).Yield 85%;mp 225 – 228°C. 1H NMR (300 MHz, DMSO-d6) *δ*: 7.52 (br, 1H), 7.40 (br, 1H), 3.95-3.93 (br, 2H), 3.5 (br, 4H), 3.11 (m, 9H), 1.49 (s, 2H), 1.23 (br, 26 H), 0.84 (br, 3H). 13C NMR (DMSO-d6, 75 MHz): 182.9, 182.3, 168.4, 166.9, 65.0, 52.7, 43.3, 37.4, 31.2, 30.6, 29.0, 28.6, 25.8, 22.0, 13.9. ESI-HRMS calcd. for C25H48N3O2 422.3759; found 422.3747 M – I+.

*2-((2-(Butylamino)-3,4-dioxocyclobut-1-en-1-yl)amino)acetic acid* (**8**).Yield 75%. mp: 217 °C (dec). 1H NMR (300 MHz, DMSO-d6) *δ*: 12.87 (s, 1H), 7.52 (s, 2H), 4.24 (s, 2H), 4.47 (s, 2H), 4.71 (m, 2H), 1.30 (m, 2H), 0.86 (t, J=7.5Hz, 3H). 13C NMR (DMSO-d6, 75 MHz): 186.17, 185.6, 174.4, (171.1, 170.8), 47.7, 46.1, 35.9, 22.2, 16.7. ESI-HRMS calcd. for C20H28N4O8Na: 475.1805, found 475.1815 2M + Na+.

*4-((2-(Butylamino)-3,4-dioxocyclobut-1-en-1-yl)amino)butanoic acid* (**9**). Yield 87%. mp: 195 °C (dec). 1H NMR (300 MHz, DMSO-d6) *δ*: 12.13 (s, 0.6H), 7.30 (s, 0.6H), 3.46 (m, 4H), 2.24 (t, *J*=6Hz, 2H), 1.72 (m, 2H), 1.48 (m, 2H), 0.87 (t, *J*=7.5Hz, 3H). 13C NMR (DMSO-d6, 75 MHz): 185.5, 177.2, 170.9, 46.1, 45.8, 36.0, 33.7, 29.4, 22.2, 16.7. ESI-HRMS calcd. for C24H36N4O8Na: 531.2431, found 531.2441 2M + Na+.

*3-(4-(N,N-dimethylamino)phenylamino)-4-hydroxycyclobut-3-ene-1,2-dione*(**10**). Yield 70%. mp: 195 °C (dec). 1H NMR (300 MHz, D2O-DMSO-d6 5%) *δ*: 7.41 (s, 4H), 3.14 (s, 6H). Anal. Calcd. for C12H12N2O3: C, 61.79 H, 5.62, N, 12.01. Found: C, 61.27 H, 5.28, N, 11.84. **10·**Na Sodium salt 1H NMR (300 MHz, D2O): 7.30 (d, *J* = 9.0 Hz, 4H), 7.02 (d, *J* = 9.0 Hz, 4H), 2.83 (s, 6H). 13C NMR (D2O, 75 MHz): 205.9, 199.2, 190.7, 180.4, 150.9, 133.1, 123.5, 119.1, 44.2. ESI-MS (negative mode): 231.09 (M–). Anal. Calcd for C12H11N2O3Na·1/2H2O: C, 54.75; H, 4.60, N, 10.64. Found: C, 54.21 H, 4.25, N, 10.73.

*3-(2-hydroxyphenylamino)-4-hydroxycyclobut-3-ene-1,2-dione*(**11**). Yield 78%. mp: 225 ºC dec. 1H NMR (300 MHz, DMSO-d6) *δ*: 9.90 (br s, 1H), 9.31 (s,1H), 7.48 (d, *J* = 8.1, 1H), 6.94, 6.87, 6.78 (m, 3H). 13C NMR (75 MHz, DMSO-d6) 186.6, 184.3, 171.4, 148.2, 125.9, 125.0, 121.6, 119.1, and 115.5. ESI-HRMS calcd. for C20H14N2O8Na 433.0648; found 433.0656, 2M+Na+.

*3-(4-carboxyethylphenylamino)-4-hydroxycyclobut-3-ene-1,2-dione*(**12**). Yield 83%. mp: >230 ºC dec. 1H NMR (300 MHz, DMSO-d6) *δ*: 10.60 (s, 1H), 7.89 (d, *J* = 8.7, 2H), 7.56 (d, *J* = 8.7, 2H), 4.27 (q, *J* = 6.8, 2 H), 1.29 (t, *J* = 6.9, 3 H). 13C NMR (75 MHz, DMSO-d6) *δ*C 189.9, 185.2, 171.2, 165.3, 143.3, 130.6, 130.4, 123.5, 118.0, 60.3, and 14.2. ESI-HRMS calcd. for calc. for C26H23N2O10 523.1353; found 523.1346 2M+H+.

*3-(4-(aminomethyl)phenylamino)-4-hydroxycyclobut-3-ene-1,2-dione*(**13**). Yield 92%. mp: >270 ºC dec. 1H NMR (300 MHz, DMSO-d6) *δ*: 9.37 (s, 1 H), 7.98 (br s, 3 H), 7.67 (d, *J* = 8.4, 2 H), 7.26 (d, *J* = 8.4, 2 H), 3.91 (2 H, d, *J* = 5.4, 2 H). 13C NMR (75 MHz, DMSO-d6) *δ*C : 202.3, 187.8, 177.4, 141.6, 129.6, 124.9, 117.2 and 42.1. ESI-HRMS calcd. for C11H10N2O3Na 467.1454; found 467.1446 M+Na+.

*3,3'-(((((methylazanediyl)bis(propane-1,3-diyl))bis(azanediyl))bis(3,4-dioxocyclobut-1-ene-2,1-diyl))bis(azanediyl))bis(N,N,N-trimethylpropan-1-aminium) iodide* (**14**). Yield 77%. 1H NMR (300 MHz, DMSO-d6) *δ*: 7.51 (br s, 1H), 3.65 (br t , 8H), 3.43 (m,4H), 3.18 (s, 18H), 2.43 (br t, J = 6.6 Hz, 4H), 2.22 (s, 3H), 2.08 (m, 4H), 2.08 (br t, J = 6.6 Hz, 4H). 13C NMR (75 MHz, DMSO-d6) *δ*C : 182.6, 182.1, 167.9, 167.6, 62.9, 54.1, 52.3; 41.7, 28.5 and 24.5. ESI-HRMS calcd. for C27H49N7O4I 662.2891 found 662.2892 M+.

*4,4'-propane-1,3-diylbis(azanediyl))bis(3-((3-dimethylamino)propylamino)cyclobut-3-en-1,2-dione*(**15**). Yield 56%. mp: 281-284 ºC. 1H NMR (300 MHz, DMSO-d6) *δ*: 7.46 (br, 4H), 3.63 (br, 8H), 2.34 (t, *J* = 6.9 Hz, 4H), 2.21 (s, 12H), 1.90 (m, 2H), 1.76 (m, 4H). 13C NMR (DMSO-d6, 75 MHz): *δ*: 182.9, 168.5, 168.1, 56.5, 45.6, 42.1, 32.9, 29.2. ESI-HRMS calc. for C21H34N6O4Na 457.2539; found 457.2537 M + Na+.

*3-(4-(aminomethyl)phenylamino)-4-hydroxycyclobut-3-ene-1,2-dione*(**16**). Yield 35%. mp >230 °C (dec.).1H NMR (300 MHz, DMSO-d6) *δ*: 8.11 (br s, 1H), 7.49–7.35 (m, 6H), 4.82 (br s, 4H), 4.05 (br s, 4H), 3.62 (t, 4H), 3.22 (s, 18H) ppm; FAB-MS (NOBA) 497.3 M−2I−H+; 625.2 M−I+.

*3-(butylamino)-4-((3-(dimetilamino)propyl)(methyl)amino)cyclobut-3-en-1,2-dione* (**17**). Yield 70%. mp 54 – 56°C. 1H NMR (300 MHz, CDCl3) *δ*:8.36 (br, 1H), 3.67 (AB, *J* = 6.3 Hz, 2H), 3.34 (s, 3H), 2.37 (m, 2H), 2.23 (s, 6H), 1.75 (m, 2H), 1.55 (m, 2H), 1.26 (m, 2H), 0.95 (t, *J* = 4.5 Hz, 3H). 13C NMR (75 MHz, DMSO-d6) *δ*: 183.7, 183.6, 169.4, 168.8, 54.5, 48.66, 45.2, 44.9, 36.5, 34.0, 24.1, 20.2, 14.2. ESI-HRMS calcd. for C14H25N3O2 268.2025, found 268.2022 M + H+.

*6,19-dimethyl-2,6,10,15,19,23-hexaazatricyclo22.2.0.011,14hexacosa-1(24),11(14)-diene-12,13,25,26-*tetraone (**macrocycle** **18**). Yield 75%. mp: > 250 °C (dec). 1H NMR (300 MHz, CDCl3) δ:8.0- 7.2 (br, 4H), 3.49 (br, 8H), 2,43 (t, J = 7.1 Hz, 8H, CH2), 2.22 (s, 6H, CH3), 1.74 (t, J = 6.2 Hz, 8H, CH2). 13C NMR (300 MHz, D2O/DCl): δ = 184.3, 170.9, 56.3, 41.8, 27.9. ESI-HRMS calcd. for C22H35N4O7S 499.2241; found: 499.2226 M+DMSO+H+.

* 1. **Parasite strain and culture**

Promastigote forms of *L. donovani* (MHOM/PE/84/LC26), *L. infantum* (MCAN/ES/2001/UCM-10) and *L. braziliensis* (MHOM/BR/1975/M2904) were cultured in vitro in medium trypanosomes liquid (MTL) supplemented with 10% inactive foetal calf serum (iFCS) in Roux flasks (Corning, USA), according to a methodology previously described (Gonzalez et al., 2005).

* 1. ***In vitro* activity assays.**

The compounds were dissolved in pure DMSO (Panreac, Barcelona, Spain) which was later diluted to a concentration of 0.1%. This concentration has been assayed and established as neither toxic nor growth-limiting for parasite and mammalian cells (Marin et al., 2011).

* 1. **Cell culture and cytotoxicity tests**

 Macrophage cells from the macrophage line J774.2 European collection of cell cultures (ECACC) number 91051511 were grown in minimal essential medium (MEM) plus glutamine (2 mM) and 20% iFCS, in a humidified 95% air and 5% CO2-atmosphere at 37ºC. The cytotoxicity test on macrophages was performed by Flow cytometric analysis according to a method previously described (Marin et al., 2011).The percentage of viability was calculated with respect to the control culture. The IC50 was calculated using linear regression analysis from the Kc values of the concentrations employed.

* 1. **Promastigote assay: extracellular forms**

 The compounds were dissolved in the culture medium at concentrations ranging from 1 µM to 100 µM. The effects of each compound against the promastigote forms at least 4 different concentrations were tested after 72 h exposure using a Neubauer haemocytometric chamber. The leishmanicidal effect was expressed as the IC50 value, i.e. the concentration required to result in 50 % inhibition, calculated by linear regression analysis from the Kc values of the concentrations employed.

* 1. **Amastigote assay: intracellular forms**

 J774.2 macrophage cells were seeded at a density of 1×104 cells/well in 24-well microplates (Nunc) with rounded coverslips on the bottom and cultured for 2 days. Adherent macrophage cells were infected with promastigotes in the stationary growth phase of *L. donovani, L. infantum* and *L. braziliensis* at a ratio of 10:1 and maintained for 24 h at 37ºC in air containing 5% CO2. Non-phagocytosed parasites were removed by washing, and the infected cultures were incubated with the testing compounds and then cultured for 72 h in MEM plus glutamine (2 mM) and 20% iFCS. Compound activity was determined from the percentage reductions in amastigote number in treated versus untreated cultures in methanol-fixed and Giemsa-stained preparations. Values are the means of three separate determinations (Marin et al., 2013).

* 1. **Infectivity assay**

 Adherent macrophage cells grown as described above, were infected in vitro with promastigote forms of *L. donovani, L. infantum* or *L. braziliensis* at a ratio of 10:1. The drugs (IC25 concentrations) were added immediately after infection and incubation was performed for 12 h at 37ºC in 5% CO2.The infected cultures were grown for 10 days in fresh medium according to (Marin et al., 2013).

* 1. **Ultrastructural alterations**

 The parasites were cultured at a density of 5×105 cells/mL in the corresponding medium, each of which contained the compounds being tested at the IC25 concentration. Cells were prepared for TEM (Zeiss model, Barcelona, Spain) using a technique previously described (Marin et al., 2011).

* 1. **Metabolite excretion**

 Cultures of *L. donovani, L. infantum* and *L. braziliensis* promastigotes (initial concentration 5×105 cells/mL) received the IC25 of the compounds (except for control cultures). After incubation for 72 h at 28ºC the cells were centrifuged (400 g/10 min) and the supernatants collected to determine the excreted metabolites by 1H-NMR, and chemical shifts were expressed in ppm, using sodium 2,2-dimethyl-2-silapentane-5-sulphonate as the reference signal. One dimensional 1H NMR spectra were acquired on VARIAN DIRECT DRIVE 400 MHz Bruker spectrometer with AutoX probe using D2O. The assignments of metabolites were based on 1D NMR spectrum. The chemical shifts used to identify the respective metabolites were consistent with those described previously by our group (Fernandez-Becerra et al., 1997). In addition, the human metabolome database (http://www.hmdb.ca/) was also used for this purpose. The spectral region from 1.0 to 5.5 ppm was bucketed into a frequency window of 0.1 ppm. The resulting integrals were normalised to the working region (1.0 - 3.4) ppm of the spectrum to correct for inter-sample differences in dilution. The binning and normalisations were achieved using Mestrenova 9.0 software. The matrix obtained in Mestrenova was imported to Microsoft Excel for further data analyses.

1. **Results and discussion**
	1. **Leishmanicidal assays**

**Tables 1**, **2** and **3** show the IC50 values obtained after 72 h of exposure for the different species of *Leishmania* (*L. infantum*, *L. braziliensis* and *L. donovani*). Toxicity values against J774.2 macrophage after 72 h of culture were also calculated, and the selectivity indices (SI = IC50 macrophages toxicity/IC50 activity of extracellular or intracellular forms of the parasite) are shown in the last two columns of **Tables 1**, **2** and **3**. Results obtained for the reference drug glucantime were included for comparison. **Table 3** shows that the leishmanicidal activities against both the extra- and intracellular forms of the *L. donovani* by the squaramides compound **1, 7, 11, 14** and **17** were, higher than those seen with Glucantime™. The same compounds also possess inhibitory properties against extra- and intracellular forms of *L. infantum* and *L. braziliensis* (**Table 1** and **2**), except compound **1** which IC50 was significantly higher than one obtained with the reference compound. However, more interesting are the toxicity data in mammalian cells, since all eighteen compounds tested were found to be much less toxic for macrophages than the reference drug, and even the most toxic among them, **15** was 2.5-fold more benign.

Toxicity values substantially influence the more informative selectivity index (SI) data, so the best values were again obtained for compounds **1, 7, 11, 14** and **17**, in tests performed on *L. donovani* specie (**Table 3**), SI exceeded that of the reference drug by 21- and 28-fold for the extra- and intracellular forms in the case of **1**, by 25- and 27-fold with **7**, by 25- and 66-fold with **11**, by 24- and 26-fold with **14** and 33- and 36-fold with **17**, while on *L. infantum* (**Table 1**), the respective values obtained were 27- and 48-fold with **7**, by 23- and 41-fold with **11**, by 24- and 35-fold with **14** and 28- and 56-fold with **17**, and in the case of *L. braziliensis* (Table 2) was 28- and 35 with **7**, by 24- and 28-fold with **11**, by 31- and 33-fold with **14** and 39- and 55-fold with **17**. It should be noted that a SI value more than 20-fold that of the reference drug is one of the usual basic criteria for considering primarily screened compounds as candidates for more advanced testing *in vitro* and in animal models (Nwaka et al., 2011).This is the reason that in our study, compound **1** has been excluded in the case of *L. infantum* and *L. braziliensis* as it misses the 20-fold of the reference drug and only been studied on *L. donovani.*

The tests described in the **Figure 3**, **4** and **5**, represent only a first crude approach to the leishmanicidal properties of the compounds assayed. In order to gain better insight into the activity of compounds **7, 11, 14** and **17** against *L. infantum* and *L. braziliensis* and in the case of *L. donovani* compound **1** was also included, their effect on the infectivity and intracellular replication of amastigotes was subsequently determined. Macrophage cells were grown and infected with promastigotes in stationary phase, in accordance with the usual working procedure. The parasites invaded the cells and underwent morphological conversion to amastigotes within 1 day after infection. On day 10, the rate of host cell infection reached its maximum (control experiment). We used the IC25 of each product as the test dosage.

As shown in **Figure 3a** and **b**, when Squaramides **7**, **11**, **14** and **17** were added to macrophages infected with *L. infantum* promastigotes, the infection rate decreased significantly with respect to the control and, furthermore, the four compounds were also more effective in decreasing infectivity than glucantime (60.8%, 55.4%, 58.1% and 74.3% for **7, 11, 14** and **17** respectively, versus only 20.0% for the reference drug). The Squaramide **17** were clearly more efficient than the remaining compounds. A measure of the average number of amastigotes per infected macrophage (**Figure 3 c** and **d**) led to similar conclusions: all four compounds were more effective than glucantime (with only a 38.6% decrease), and their order of effectiveness followed the same pattern as that seen in the infectivity measures, although the differences between them were less pronounced (50.7%, 56.8%, 54.4% and 62.7% for **7, 11, 14** and **17**, respectively).

The same experiment was performed with *L. braziliensis*, and the results obtained concerning infection rates (a and b) and amastigote numbers (c and d) can be observed in Figure **4**. In both cases, the four compounds were also more effective than glucantime, and the order of effectiveness was now **17 > 11 > 14 > 7**, since the infectivity rates calculated from **Figure 4** (**a** and **b**) were: 72%, 62%, 52%, 49% and 15%; and the decrease in amastigote numbers was: 62%, 51%, 47%, 43% and 16% for **17, 7, 14, 11** and Glucantime™ respectively **Figure 4** (**c** and **d**).

In the case of *L. donovani*, the results concerning to infection rates (**a** and **b**) and amastigotes numbers (**c** and **d**) can be observed in **Fig. 5**. As was happening in the case of *L. infantum* and *L. braziliensis*, all compounds tested were more effective than Glucantime™ and also in both cases the order of effectivenes was very similar: **17 > 11 > 7 > 14 > 1**, since the infectivity rates calculated from **Fig. 5** (**a** and **b**) were: 59%, 52%, 45%, 43%, 38% and 15%; and the decrease in amastigote numbers was: 64%, 53%, 51%, 49%, 45% and 16% for **17, 11, 7, 1, 14** and Glucantime™ respectively **Fig. 5** (**c** and **d**).

Now, if we compare the results obtained for three *Leishmania* species, it could be concluded that, in accordance with the SI values displayed in **Tables 1, 2** and **3** and their effect on the infectivity and intracellular replication of amastigotes **Figures 3, 4** and **5**, the squaramide **17** which was presented greater leishmanicidal effectiveness against the three species studied.

At this point it is hard to correlate these results with any structural feature except for the presence of the squaramide unit. For instance, the lipidic squaramide **7** could be considered as a miltefosine (MF) analogue due to the bioisosteric replacement of the phosphate group of MF by a squaramide unit **(Figure 6)**, thus suggesting a possible mechanism (Sato et al., 2002).On the contrary, squaramide **11** showing similar leismanicidal effect, is an small anionic compound totally ionized in solution. Remarkably, the most active compound in this series is the neutral squaramide **17** that also resulted very effective against *T. cruzi* (Olmo et al., 2014).

* 1. **Ultrastructural alterations**

Since all these compounds (**1, 7, 11, 14** and **17**) showed remarkable leishmanicidal activity, major damage to parasite cells could be inferred. In order to confirm this assumption, a transmission electron microscopy (TEM) study on the promastigote forms of the three *Leishmania* species under consideration was performed. As expected, significant morphological alterations were observed compared with untreated control cells. **Figures 7, 8** and **9** displays some of the most relevant structural features observed in control and treated cells of promastigotes of the three species, which are commented on below.

Ultrastructural alterations of *L. infantum* treated with compounds **7, 11, 14** and **17** by TEM are showed in **Figure 7** (**plates B-F**) and compared to the untreated control sample (**Figure 7, plate A**). In all cases, there was a strong general vacuolization present after the exposure of promastigotes to the selected compounds. This effect can be primarily seen in **Figure 7, plate B** (Squaramide 7). These vacuoles were enormous, in some cases they were filling the whole cytoplasm and they were either hollow or full of unrecognizable structures. The parasites were lacking organelles and, especially, ribosomes, indicating a state very close to cell death. The mitochondria were frequently swollen. Some parasites were adopting strange star-like cellular forms. On the contrary, the cytoplasm of promastigotes treated with compound **17** (**Figure 7, plate C**) appeared quite healthy and the ribosomal content was, also, normal. However, the effectiveness of this compound was brought to light by the increased vacuolization and by the notable strong dilatation of the nuclear membrane (arrow). The aspect of some parasites treated with the squaramide **14** (**Figures 7, plates D-E**) was unrecognizable. One of the damaged cellular structures was the plasmatic membrane. Its outline was irregular and in addition, the microtubular structure was not present (arrow). The aspect of the nucleus and cytoplasm was similar to the other cases, and given the same tone of the cytoplasm, it was impossible to clearly distinguish other organelles, if they were present. We could see some different-sized vacuoles, some of which were empty and others were filled with small electron-dense vesicles. In some, less damaged parasites, we could see flagella, mitochondria or glycosomes, but these cellular structures were extremely swollen. Compound 11 has been responsible for the near-death aspect of many parasites visible in the ultra-thin cuts. In **Figure 7, plate F** the cytoplasm of the parasites was deficient in ribosomes and possessed plenty of empty vacuoles or, again, electron-dense aggregates. In some, we saw a swollen mitochondrion, retracted nucleus and dilatation of the nuclear membrane.

The ultrastructural alterations caused by compounds **7, 11, 14** and **17** against *L. braziliensis* are shown in **Figure 8** (**plates B-F**) and are compared to the control sample (**Figure 8, plate A**). Out of these 5 compounds, the most noticeable alterations were produced by the compound **7** (**Figure 8,** **plates B-C**). Once more, the promastigotes were lacking ribosomes; the cytoplasm was packed with vacuoles and, in some occasions, the mitochondria were unrecognizable, swollen and their crista were distended and had aspect of little vacuoles (**Figure 8, plate B**). Other parasites were full of lipid vacuoles (**Figure 8, plate C**) or a great amount of glycosomes (**Figure 8, plate B**). The Golgi apparatus was also swollen and disorganised in the vast majority of the parasites. In **Figure 8, plate B** we see a damaged nucleus with completely disorganised chromatin. The compound **11** (**Figure 8, plate D**) also caused swelling and vacuolization of the parasites. These vacuoles appeared in both damaged and healthy looking parasites and were either empty or full of myelin agglomeration. The ribosome deficiency has been quiet normal in the majority of treated promastigotes. In the case of the compound **14**, it led to the death of many promastigotes of *L. braziliensis* and, even in the ones that were still alive, were smaller in size (arrow) and packed with empty or lipid vacuoles that were filling the cytoplasm, considering it has not any plasmatic organelles in it (**Figure 8, plate E**). In some, there have been disfigured and fragmented mitochondria. The promastigote form of *L. braziliensis* resulted wrecked after the exposure to the compound **17** (**Figure 8, plate F**) and the kinetoplast and mitochondrion were swollen. The intense vacuolization and lack of ribosomes gave the cytoplasm a clear and hollow aspect. Abundant glycosomes occupy a major part of the cytoplasm in other parasites.

The ultrastructural alterations caused by the same 4 compounds (**7, 11, 14, 17**) plus compound **1** against promastigotes *L. donovani* were studied at TEM. The effectiveness of these compounds is shown in **Figure 9** (**plates B-F**) and compared to the control in **Figure 9, plate A**.

All these compounds have altered the typical structure of the promastigotes in a great extent. The compound **17** affected the morphology of parasites (**Figure 9, plate B**), breaking the plasmatic membrane into pieces or, in the majority of cases, making it undulating, which is completely abnormal. The internal content does not look nothing like the control sample, in view of the vacuoles of different types present in the cytoplasm. Some of these vacuoles were large lipid vacuoles, other were empty and some were strange and enormous, full of highly electron-dense punctiform material or even smaller vesicles. The electron-dense nuclear material was separated from the nuclear membrane and, equally, there was electron-dense material present in the glycosomes. The mitochondria were swollen. We show, in **Figure 9, plate C**, an example of many ultrastructural images of *L. donovani* promastigotes exposed to the compound **9**. These parasites possessed a completely damaged and irregular plasmatic membrane without microtubules (arrow). In the cytoplasm, deficient in ribosomes, there were three types of vacuoles: empty, lipid and excessively big ones. The latter have possibly derived from the degeneration of the kinetoplast and were full of smaller vacuoles. The kinetoplast was swollen to the point of rupture and had a helicoid, disorganised structure. The electron-dense glycosomes and the expanded mitochondria were almost in every case associated to each other. In the background, there were rests of flagella indicating previous massive cellular death. The contact of the compound **11** with promastigotes resulted, apart from dead parasites (**Figure 9 plate D**), in reduced viability, very little ribosomes, enormous and highly electron-dense glycosomes and multiple vacuoles, many with apparent rests of kinetoplast. On some occasions, there were membranous structures involving the distended mitochondria (arrow). Compound **1** (**Figure 9, plate E**) similarly altered the *L. donovani* promastigotes, but now the ribosomes were equally abundant as in control samples and it seemed that they suffered a rapid death, rather than a gradual degenerative process. The damages were limited to only a few cell structures and this gave the impression as if the promastigotes exposed to a fixative agent. The plasmatic membranes were very electron-dense and scalloped (arrow), and there was an intense vacuolization as in previous compounds. The vacuoles were either large and empty or filled with little electron-dense spots, or huge, originated in the degeneration of mitochondria and kinetoplasts. Compound **14** (**Figure 9, plate F**) caused similar alteration as described above: enlarged promastigotes, without microtubular structure in the plasmatic membrane and flagella ruptured into two pieces. Cytoplasm was packed with vacuoles of various types and sizes. Some were small, other were large and empty resembling degraded mitochondria full of smaller vesicles that probably have been mitochondrial crests. Also, the glycosomes had supremely electron-dense content, which did not look like the content of untreated, control samples.

* 1. **Metabolite excretion**

Since trypanosomatids are unable to completely degrade glucose to CO2, they excrete a considerable portion of its hexose skeleton as partially oxidized fragments in the form of fermented metabolites, whose nature and percentage depend on the pathway used for glucose metabolism (Ginger, 2005; Bringaud et al., 2006). The catabolism products in *Leishmania* are usually CO2, succinate, acetate, pyruvate, D-lactate, L-alanine, and, to a lesser extent, ethanol (Cazzulo, 1992). Detection of large amounts of succinate as a major end product is a usual feature, because it rules on glycosomal redox balance, enabling reoxidation of NADH produced in the glycolytic pathway. Succinic fermentation requires only half of the phosphoenol pyruvate produced to maintain the NAD+/NADH balance, and the remaining pyruvate is converted inside the mitochondrion and the cytosol into acetate, D-lactate, L-alanine or ethanol according to the degradation pathway followed by each species (Michels et al., 2006).

In order to obtain some information concerning the effect of the tested compounds on glucose metabolism, we registered the 1H-NMR spectra of MTL media in which *Leishmania* spp. promastigotes were grown for 3 days with treatment or without it (control), and the final excretion products were identified qualitatively and quantitatively (Supplementary data 1). The characteristic presence of acetate, D-lactate, succinate, L-alanine, and small amounts of pyruvate, was confirmed in the control experiments performed on the three species of *Leishmania*.

As expected, succinate and acetate were the most abundant end products identified. However, after treatment with **1, 7, 11, 14** and **17**, the excretion of catabolites was substantially altered at the dosages employed. **Figure 10** displays these modifications with respect to the control observed at the height of the spectral peaks corresponding to the most representative final excretion products. Strong differences in the catabolic pathway appeared, and that seemed to be connected with the leishmanicidal activity commented on above. The main features found for *L. infantum* was substantial increases in the formation of succinate with the compound **7**, accompanied by increases in the formation of acetate, D-Lactate and piruvate. Contrary, to the metabolite effect of compound **14** which had a marked inhibition on the metabolite excretion (succinate, acetate and piruvate). If we turn now to the results obtained with the *L. braziliensis* species, compound **7** also substantially increased the formation of succinate, and decreased the production of the others metabolites, and compound **17** decreased all metabolite excretion. In the case of *L. donovani*, succinate excretion was increased by compound **1, 7** and **14**. All these data could be interpreted on the basis of a change in the succinate and acetate pathways occurring in the presence of the most active compounds. It is well known that acetate, D-lactate, L-alanine and ethanol originate from the transformation of PEP in pyruvate in the presence of pyruvate kinase or pyruvate phosphate dikinase (Bringaud et al., 2006). Therefore, it seems possible that compounds were interacting with the pyruvate kinase enzymes and modifying the glucose metabolism of the parasite at the pyruvate stage.

Those catabolic changes could also be related to a malfunction of the mitochondria, due to the redox stress produced by inhibition of the mitochondrion resident Fe-SOD enzyme (Kirkinezos et al., 2001) which should result in decrease of pyruvate metabolism and a consequent decrease of the succinate produced in mitochondria. These data appear to confirm that the severe modifications generated in organelles such as glycosomes or mitochondria by the compounds are the ultimate reasons for the alterations observed in the excretion products of the three species.

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1. **Transparency declarations**

None to declare

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