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Betulin derivatives impair *Leishmania braziliensis* viability and host–parasite interaction

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ABSTRACT

Leishmaniasis is a public health problem in tropical and subtropical areas of the world, including Venezuela. The incidence of treatment failure and the number of cases with Leishmania-HIV co-infection underscore the importance of developing alternative, economical and effective therapies against this disease. The work presented here analyzed whether terpenoids derived from betulin are active against New World *Leishmania* parasites. Initially we determined the concentration that inhibits the growth of these parasites by 50% or IC_{50}, and subsequently evaluated the chemotactic effect of four compounds with leishmanicidal activity in the sub-micromolar and micromolar range. That is, we measured the migratory capacity of *Leishmania* (*V.*) *braziliensis* in the presence of increasing concentrations of compounds. Finally, we evaluated their cytotoxicity against the host cell and their effect on the infectivity of *L.* (*V.*) *braziliensis*. The results suggest that (1) compounds **14**, **17**, **18**, **25** and **27** are active at concentrations lower than 10 μM; (2) compound **26** inhibits parasite growth with an IC_{50} lower than 1 μM; (3) compounds **18**, **26** and **27** inhibit parasite migration at pico- to nanomolar concentrations, suggesting that they impair host–parasite interaction. None of the tested compounds was cytotoxic against J774.A1 macrophages thus indicating their potential as starting points to develop compounds that might affect parasite–host cell interaction, as well as being leishmanicidal.

1. Introduction

Failure of environmental vector control measures, lack of education and training in communities, as well as reports of treatment failures justify the drive involved in the design and analysis of compounds with therapeutic potential against *Leishmania*. For example, understanding the influence of chemotaxis during the infective process might underscore important strategies for developing new, better and more effective treatment options.

Promastigote flagellum plays a fundamental role in the translation and attachment of parasites to host epithelium.[1,2] This process is promoted by chemical signals that modulate parasite behavior and are essential for its survival in the skin.[3] In fact, microorganisms evaluate their surroundings and move toward the most attractive, avoiding toxic compounds. Chemotaxis, however, is not the only factor that triggers migration, as it also occurs in response to changes in hydrostatic pressure, light levels, magnetic fields, osmotic pressure, temperature, etc.[4] All this indicates that the comprehension of processes involved in chemotaxis are essential for understanding the behavior of migrating cells, such as *Leishmania*.

Chemotaxis is the key event that initiates the successful interaction between parasite and host; it involves mutual recognition and migratory responses that determine the infection.[5] It would therefore be desirable to identify compounds which prevent host–parasite interaction before phagocytosis occurs with the subsequent evolution of the promastigote into amastigote. If successful, prevention of parasite entry into the host cells would impair subsequent infection and successful installation of the disease.

The present work describes the leishmanicidal activity and chemotactic activity of betulin derivatives. Betulin (lup-20(29)-ene-3β,28-diol) is a triterpene abundant in birch (*Betula* sp. L.) bark.[6] Derivatives of betulin and its closely related oxidation product, betulinic acid have been described for their anti-inflammatory

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and cytotoxic activity against certain cancer cell lines,[6,7] as well as for their activity inhibiting vascular smooth muscle proliferation and migration.[8] Their main mechanism of action has been related to programmed cell death.[9] Their biological activity includes a variety of pharmacological effects against parasites, among them, *Leishmania donovani* and *Leishmania tropica* (IC_{50} = 14.6 μM).[10–14] Their proposed mechanism of action has been the inhibition of topoisomerase activity and apoptosis induction in *L. donovani*.[11,15–17] Conspicuous is that in the case of drug-resistant parasites, structurally related betulin derivatives can efficiently reduce the load of parasites that infect the host cell, without affecting the viability of the macrophage. Additionally, betulinic acid derivatives are active against *Plasmodium* spp. resistant to chloroquine.[18] This places triterpenoid betulin derivatives as a possible therapeutic option that demands further analysis of their action.

Herein, we describe the leishmanicidal activity of a group of betulin derivatives in vitro. Based on the obtained results we selected compounds with improved activity and assayed their chemotactic activity and their cytotoxicity and effect on the infectivity of the macrophage cell line J774.A1. The results suggest their potential activity as starting points to develop leishmanicidal agents, also impairing host–parasite interaction.

2. Materials and methods

2.1. General procedures

Commercially available reagents were used without further purification and all of the solvents were of HPLC grade. Anhydrous solvents were purchased from Sigma–Aldrich. All reactions in anhydrous solvents were performed in oven dried glassware under an inert atmosphere of anhydrous argon or nitrogen. Thin layer chromatography (TLC) was performed on E. Merck Silica Gel 60 aluminium packed plates, with visualization accomplished by UV illumination and staining with 5% H_2SO_4 in MeOH. The ^{1}H NMR spectra were measured on a Varian Mercury-VX 300 MHz or a Chemagnetics CMX 400 MHz spectrometer with chemical shifts reported as parts per million (in $CDCl_3$ at 23 °C, solvent peak at 7.26 ppm as an internal standard, or in DMSO-d_6 at 23 °C, solvent peak at 2.50 ppm as an internal standard). The ^{13}C NMR spectra were obtained on a Varian Mercury-VX 75 MHz or a Chemagnetics CMX 100 MHz spectrometer with chemical shifts reported as parts per million (in $CDCl_3$ at 23 °C, solvent peak at 77.0 ppm as an internal standard, or in DMSO-d_6 at 23 °C, solvent peak at 39.50 ppm as an internal standard). Elemental analyses were performed to determine purity (>95%) of all tested compounds. Elemental analyses were performed by Robertson Microlit Laboratories, Madison, NJ, USA. Melting points were obtained with a Sanyo Gallenkamp apparatus without correction. The Fourier transform infrared (FTIR) spectra were recorded on a Bruker Vertex 70 spectrometer with Pike MIRacle™ diamond crystal or with a Bruker Equinox 55 spectrometer including IR Scope II and diamond anvil.

2.2. Betulin derivatives

A collection of 28 betulin-derived triterpenoid compounds belonging to two different structural subfamilies, simple betulin derivatives (20 compounds) and heterocyclic triazolodione derivatives (8 compounds), were tested for their leishmanicidal activity. The betulin derivatives were prepared according to the previously described procedures,[11,19,20] except for three novel compounds **26**, **27** and **28**. Allobetulin acetate **19** was synthesized according to the literature procedure[21] and spectral data was identical to that reported in the literature.[22] Heterocyclic betulin intermediate (**HC-B**, Scheme 1), which was used as a starting material for the preparation of new derivatives **26**–**28**, was synthesized by the published method.[11] Briefly, betulin was converted in four steps to 3,28-di-*O*-acetyllupa-12,18-diene. This compound was then reacted in the Diels–Alder reaction with 4-methyl-1,2,4-triazoline-3,5-dione and finally, its acetyl groups at C3 and C28 hydroxyls were removed by the treatment with aqueous sodium hydroxide to give the heterocyclic betulin intermediate **HC-B**. The heterocyclic derivative **26** with small formyl groups at C3 and C28 was produced in 83% yield by refluxing the starting compound **HC-B** in formic acid. Heterocyclic betulinic aldehyde derivative **27** was obtained in 86% yield by oxidizing **HC-B** with tetra-*n*-propylammonium perruthenate (TPAP) catalyst in the presence of oxygen. Heterocyclic betulonic aldehyde derivative **28**, in turn, was synthesized in 67% yield by oxidizing **HC-B** with excess of pyridinium chlorochromate (PCC) in dichloromethane.

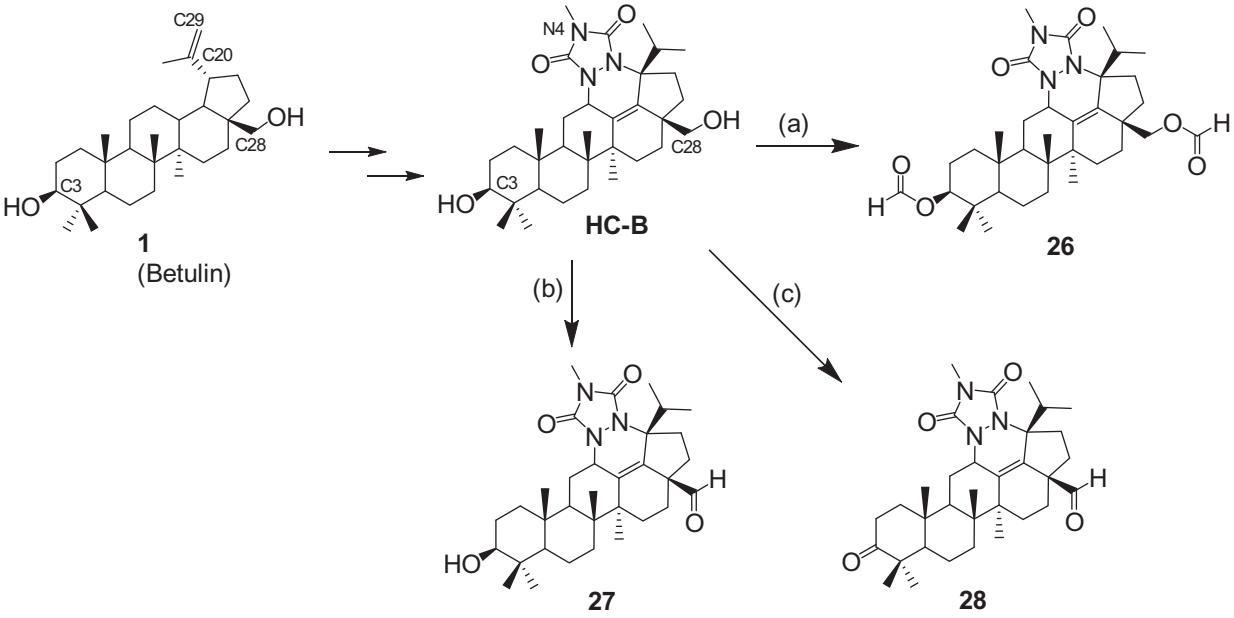
The compounds are stable at room temperature, were dissolved in DMSO at 10 mM concentration and were stored at −20 °C until use. Supplementary material (Table S1) presents a summary of the derivatives, their formulas and their macroscopic characteristics when dissolved in DMSO. All but one compound were colorless, two compounds produced cloudy solutions, three formed a precipitate and four produced a suspension. These properties were important to describe as they could impair the compounds' antileishmanial effect.

2.2.1. 3,28-Diformylbetulin-derived heterocyclo adduct with 4-methyl-1,2,4-triazoline-3,5-dione (26)

A mixture of betulin heterocycle **HC-B** (0.20 g, 0.36 mmol) and formic acid (85%, 6 mL) was stirred at under reflux for 2.5 h. Water (36 mL) was added, and the resulting mixture filtered. Removal of the solvent in vacuo gave the product **26** (185 mg, 83%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.89 (s, 3H), 0.90 (s, 3H), 0.93 (s, 3H), 1.03 (s, 3H), 1.06 (s, 3H), 3.04 (s, 3H), 4.07 (m, 2H), 4.64 (m, 2H), 8.11 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 18.7, 18.9, 20.5, 21.5, 21.7, 22.7, 24.7, 25.3, 25.7, 27.3, 28.2, 30.2, 31.2, 35.4, 35.9, 36.1, 39.6, 39.9, 40.2, 40.3, 40.8, 45.4, 45.8, 51.8, 55.0, 58.1, 69.4, 75.2, 83.0, 138.4, 141.5, 151.0, 153.0, 163.4, 163.4; FTIR (ν, cm^{-1}) 2945, 2876, 1752, 1724, 1697, 1467, 1392, 1367, 1335, 1265, 1177, 1143, 1029, 1009, 962, 947, 922, 906, 748; MS $[M]^{+\cdot}$ 609 m/z, 11% (9.26 min); Elemental analysis ($C_{35}H_{51}N_3O_6$) C, H, N: calcd: 68.94, 8.43, 6.89, measured: 68.77, 8.50, 6.38; mp 170 °C; R_f 0.6 (ethyl acetate/hexane 5:1).

2.2.2. Betulinic aldehyde-derived heterocyclo adduct with 4-methyl-1,2,4-triazoline-3,5-dione (27)

A mixture of betulin heterocycle **HC-B** (150 mg, 0.27 mmol) and powdered 4 Å molecular sieves (255 mg) in dry dichloromethane (16 mL) was stirred at room temperature under oxygen atmosphere for 15 min. When tetra-*n*-propylammonium perruthenate (9.7 mg, 0.030 mmol) was added, solution turned black. Reaction was carried out at room temperature under oxygen atmosphere for 6 d. The resulting mixture was filtered through a pad of Celite (7.5 cm) and rinsed with dichloromethane. Removal of the solvent in vacuo gave the crude product, which was purified by column chromatography on aluminium oxide (1:2 → 8:1 ethyl acetate/hexane) to yield the compound **27** (128 mg, 86%) as an off-white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.78 (s, 3H), 0.95 (s, 3H), 0.96 (s, 3H), 0.97 (s, 3H), 0.99 (s, 3H), 1.08 (s, 1H), 3.02 (s, 3H), 3.19 (m, 1H), 4.71 (m, 1H), 9.53 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 15.1, 16.5, 18.1, 18.5, 18.6, 20.2, 22.5, 22.7, 24.8, 26.0, 26.9, 27.9, 28.1, 29.0, 33.7, 34.1, 35.6, 37.5, 37.9, 38.5, 38.8, 42.6, 49.4, 52.5, 54.4, 55.6, 71.0, 78.7, 135.1, 136.1, 148.4, 150.1, 201.1; FTIR (ν, cm^{-1}) 3450, 2959, 2936, 2871, 2245, 1752, 1724, 1691, 1470, 1392, 1372, 1337, 1272, 1206, 1187, 1140, 1118, 1077, 1048, 1036, 1027, 1004, 919, 826, 730; MS $[M]^{+\cdot}$ 551 m/z, 9% (6.97 min); Elemental analysis ($C_{33}H_{49}N_3O_4 \times 0.67$EtOAc) C, H, N:



Scheme 1. Synthesis of novel heterocyclic betulin derivatives (**26**, **27** and **28**). Reagents and conditions: (a) HCOOH (excess), reflux at 70 °C, 2.5 h, 83%. (b) 10% TPAP, O_2, CH_2Cl_2, 4 Å molecular sieves, rt, 6 d, 86%. (c) PCC (5.8 equiv), CH_2Cl_2, rt, 3.5 h, 67%.

calcd: 70.17, 8.97, 6.89, measured: 69.89, H 7.86, N 7.13; mp 186 °C; R_f 0.2 (ethyl acetate/hexane 5:1).

2.2.3. Betulonic aldehyde-derived heterocyclo adduct with 4-methyl-1,2,4-triazoline-3,5-dione (28)

A mixture of betulin heterocycle **HC-B** (201 mg, 0.36 mmol) and pyridinium chlorochromate (460 mg, 2.1 mmol) in dry dichloromethane (68 mL) was stirred at room temperature under nitrogen atmosphere for 3.5 h. Diethyl ether (25 mL) was added, and the resulting mixture was filtered through a pad of aluminium oxide (3 cm) and rinsed with a 9:1 mixture of dichloromethane and methanol (250 mL). Removal of the solvents in vacuo gave the crude product, which was purified by column chromatography on aluminium oxide (1:1 ethyl acetate/hexane) to yield the compound **28** (133 mg, 67%) as an off-white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.06 (s, 3H), 1.07 (s, 3H), 1.10 (s, 3H), 1.12 (s, 3H), 1.14 (s, 3H), 3.04 (s, 3H), 4.75 (m, 1H), 9.55 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 15.8, 18.6, 18.7, 19.4, 20.0, 20.9, 22.7, 24.8, 26.1, 26.4, 28.0, 29.1, 33.1, 33.6, 34.2, 35.7, 37.9, 38.0, 38.3, 42.7, 47.5, 48.9, 52.4, 54.5, 55.2, 71.1, 135.5, 135.8, 148.5, 150.2, 201.0, 216.9; FTIR (ν, cm^{-1}) 2988, 2936, 2874, 2854, 1748, 1694, 1471, 1396, 1383, 1333, 1275, 1264, 1212, 1140, 1109, 1041, 1010, 992, 881, 826, 747, 709; MS [M]$^{+\cdot}$ 549 *m*/*z*, 7% (12.25 min); Elemental analysis ($C_{33}H_{47}N_3O_4 \times 0.40$EtOAc) C, H, N: calcd: 71.05, 8.65, 7.18, measured: 71.13, 8.32, 7.12; mp 245 °C; R_f 0.3 (ethyl acetate/hexane 5:1).

2.3. Strains and culture conditions

The reference strain *Leishmania* (*V.*) *braziliensis* (MHOM/BR/LTB300) was kindly provided by Dra. Noris Rodríguez (Instituto de Biomedicina, Universidad Central de Venezuela). *Leishmania* promastigotes were grown at 26 °C in semisolid blood agar supplemented with glucose–NaCl medium (glucose 1.5%, NaCl 0.85%, weight: vol) until used. Promastigotes at late log growth phase were collected by centrifugation at 1500×*g* for 10 min at room temperature. The medium was decanted and the cells were suspended in the appropriate buffer according to the experiment to be performed.

J774.A1 murine macrophages were donated by Dr. Concepción Hernandez (Instituto de Biología Experimental, Universidad Central de Venezuela). Cells were grown in complete RPMI 1640 medium, containing 10% FCS, 2 mM L-glutamine, and streptomycin 0.16 mg mL^{-1} (complete medium) at 37 °C, 5% CO_2 atmosphere. Cells were harvested at confluence.

2.4. Experimental design

2.4.1. Effect of betulin derivatives on *L. braziliensis* viability

L. braziliensis promastigotes (1×10^7 cell mL^{-1}) were seeded and cultivated at 26 °C for 5 consecutive days in semisolid medium as previously described. Betulin derivatives were added at increasing concentrations 24 h after starting the experiment. Alive parasites were enumerated for the next 72 h and the cell density was estimated with a haemocytometer.[23,24]

2.4.2. Effect of betulin derivatives on the growth and viability of host cells

J774.A1 macrophages were mechanically detached from the bottom of the culture flasks at 4 °C; the detached cells were washed with 50 mM phosphate buffer saline (PBS, pH 7.2, 0.9 M NaCl) and re-suspended in complete medium at a density of 2×10^6 cells mL^{-1}. Cells (200 μL per well of a 96-well chamber) were then seeded and cultivated at 37 °C and 5% CO_2 for 30 min to ensure the adherence of viable cells. Test compounds were then added at increasing concentrations. Control (untreated and DMSO-treated) wells were always included in the experiment. After 24 h incubation 20 μL of Alamar Blue® was added. The plate was then incubated at 37 °C and 5% CO_2 for 4 h and the development of color was then measured at 550 and 630 nm according to the protocol. A second measurement was made at 48 h as the end point of the protocol.

2.4.3. Effect of betulinic acid derivatives on chemotaxis in *L. braziliensis*

The chemotactic response of *L. braziliensis* promastigotes was determined by a modification of the two-chamber capillary assay.[25] Briefly, the tips of an 8-channel-micropipette were used as the inner chamber and the wells of a 96-well plate were used as outer compartments of the two-chamber system. The tips were filled with the experimental substance (100 μL) control or

increasing concentrations of betulin derivatives, 10^{-12}–10^{-6} M). The wells were filled with the *L. braziliensis* suspension (200 μL, 4×10^7 cells mL^{-1}). The cells were incubated for 30 min. At the end of the incubation time the cells that migrated into the inner chamber were fixed in formaldehyde (2%) diluted in 50 mM PBS (pH 7.2, 0.9 M NaCl). The cells were then counted in a haemocytometer.[25]

2.4.4. Effect of betulin derivatives on macrophage infection

J774.A1 macrophages were isolated and seeded as previously described. After 30 min of seeding, non-adherent cells were removed by washing with RPMI. The adherent macrophages were infected with stationary phase *L. braziliensis* promastigotes at a 10:1 ratio parasite/macrophage. To analyze whether the compounds affect host-cell–parasite interaction, the selected betulin derivatives were added simultaneously and remained for the rest of the incubation time. Amphotericin-B, a leishmanicidal compound, was used as a positive control. DMSO (0.5%) was used as a solvent control. After 4 h of incubation the cells were stained by Hemacolor® using a standard protocol. The percentage of infected cells and the average number of adherent or intracellular parasites was determined by manual counting at least 300 macrophages using light microscopy.

2.5. Statistical analysis

Data on drug susceptibility is expressed as mean values ± standard error of the mean (SEM) of at least five experiments. To determine the concentration that decreases the growth of parasites by 50% (IC_{50}), we used Interpolation[26] and Probit analysis.[27] We performed this analysis with the aid of the SPSS Statistics 20 and Microsoft Excel 2010, as well as the Trimmed Spearman-Karber software from the United States Environmental Protection Agency. Data on chemotaxis is expressed as mean ± SEM of the number of cells that migrated to the inner chamber (n = at least 4 experiments for each betulin derivative). The statistical significance of differences between cells exposed to betulin derivative and control cells, of in migrating cells, macrophage cytotoxicity (n = at least 3 experiments) and macrophage infectivity assays (n = at least 4 experiments) was performed by Student's t test using the GraphPad Prism-5© software.

3. Results

3.1. Effect of betulin derivatives on the *L. braziliensis* viability

Susceptibility of *L. braziliensis* promastigotes to betulin derivatives was evaluated on parasite growth. Figure 1 demonstrates the percentage of growth of *L. braziliensis* at two concentrations (100 and 10 μM) of each compound. The effect was tested 72 h after addition of the compounds. Pentamidine was used as positive control. Derivatives **14**, **17**, **18**, **25**, **26** and **27** decreased parasite growth more than 50% even at 10 μM. For these compounds, dose–response curves were constructed to accurately determine their IC_{50}. The results illustrated in Figure 2 demonstrate that five of the six derivatives displayed IC_{50} lower than 5 μM, suggesting their potential therapeutic characteristics.

3.2. Effect of betulin derivatives on the growth and viability of host cells

Based on these results we then evaluated the capacity of compounds to inhibit J774.A1 macrophage proliferation. Compound **17** was not included in the analysis since its IC_{50} against *L. braziliensis* was higher than 5 μM. Compound **25** was not further tested due to the appearance of particles in the stock solution. None of the other terpenoids decreased significantly macrophage growth, evaluated at 10 μM concentrations (data not shown). Results on cytotoxicity of the tested betulin derivatives are summarized in Table 1. That is, compounds IC_{50} against parasites, as well as their cytotoxicity against macrophages. The selectivity index (SI) was also calculated. Results listed in Table 1 demonstrate that compounds are selective against parasites, the best compound being **26** with a SI of 40.

3.3. Effect of betulin derivatives on chemotaxis in *L. braziliensis*

Next we studied the effect of compounds **17**, **18**, **26** and **27** on the taxis of *L. braziliensis*. Figure 3 illustrates the results obtained on parasite migration at increasing concentrations of the chosen compounds, as a result of the taxis exerted by them on the cell. The chemotactic experiments lasted 30 min to specifically measure chemotactic effects.[25] Figure 3 displays the data at increasing concentrations from 10^{-12} to 10^{-7} M for **17**, **18** and **27** and from 10^{-12} to 10^{-8} for **26**. Data obtained at higher concentrations could represent mixed tactic and leishmanicidal effects and for clarity are not included in figure. Of note, in chemotactic experiments (30 min) performed at 10^{-6} M, the percentage of dead cells enumerated in the outer chamber was 66% for compound **17**. This percentage remained within 25% and 12% for compounds **18**, **26** and **27**. Betulin derivatives **18** and **26** exerted a dose-dependent negative chemotactic effect, whereas compounds **17** and **27** exerted a small effect that remained similar through the concentration range tested. These data demonstrate that betulin triterpenoids produce a negative chemotactic effect on the parasites, decreasing the percentage of cells migrating to the inner chamber up to 50% (**26**) and 70% (**18**) compared to control. More interestingly, these data indicate that the effect of compounds **18** and **26** occurs at concentrations one order of magnitude lower than their respective IC_{50} towards parasites thus suggesting that **18** and **26** might affect parasite–host cell interaction, being leishmanicidal, at higher concentrations.

3.4. Effect of betulin derivatives on macrophage infection

Interaction between parasite and host cell is essential for infection. In the search for compounds that could be effective in this crucial step, herein we explored the effect of betulin derivatives capable of inhibiting parasite migration, in the internalization of *L. braziliensis* by macrophages. Due to their lower IC_{50} towards promastigotes and their differential effect on parasite migration, the chosen compounds were **26** and **27**. The data from Table 2 demonstrates that after 4 h incubation the percentage of parasites that interact and adhere to macrophages is not affected either by amphotericin-B or by compounds **26** and **27**. However, the percentage of infected macrophages substantially decreased relative to control (20%) similar to the decrease in infection rate found in infected cells treated with amphotericin-B. In Table 3, only infected macrophages are analyzed. The data demonstrate that the mean number of parasites per cell did not change between control conditions and amphotericin-B treated macrophages. However, compound **26** selectively decreased the percentage of infected macrophages with more than 10 parasites per cell and compound **27** decreased the percentage of infected macrophages containing 5–10 and more than 10 parasites per cell. Of note, these effects are observed at concentrations down to three orders of magnitude for **27** and two orders of magnitude for **26** lower than their IC_{50} against promastigotes suggesting that at these low concentrations far from those exerting their leishmanicidal effect, these compounds prevent the efficient host cell–parasite interaction and installation of *Leishmania* in the host cell.

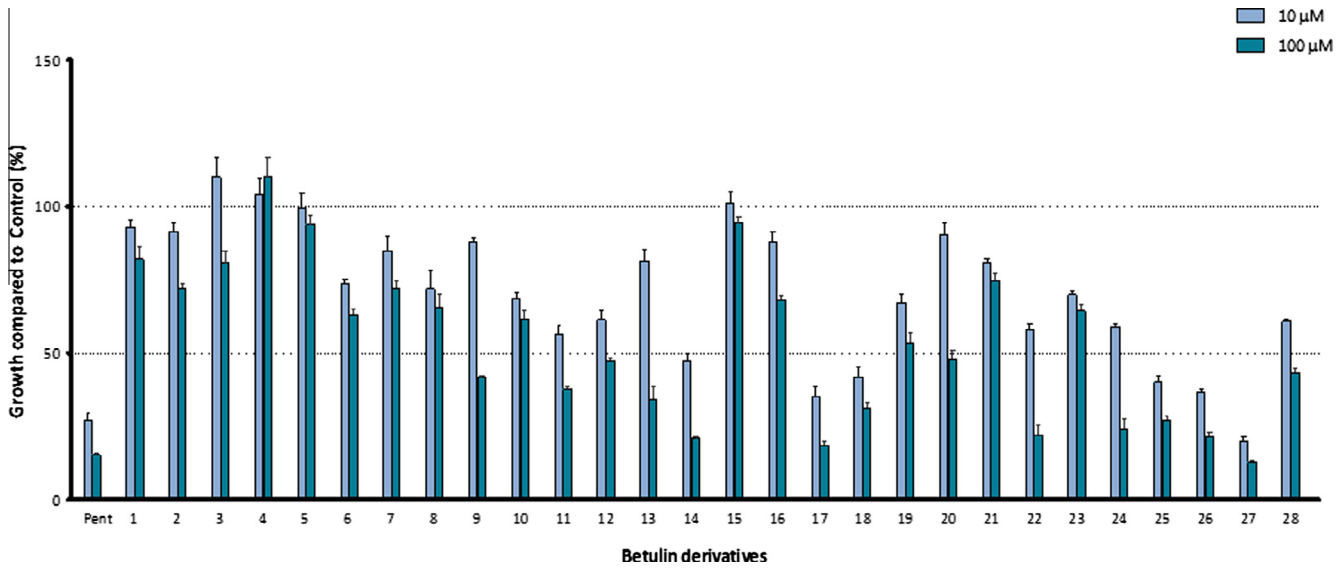


Figure 1. Inhibition of growth of *L. braziliensis* evaluated after 72 h incubation. Results represent the mean ± SEM of three independent experiments. Positive control, pentamidine.

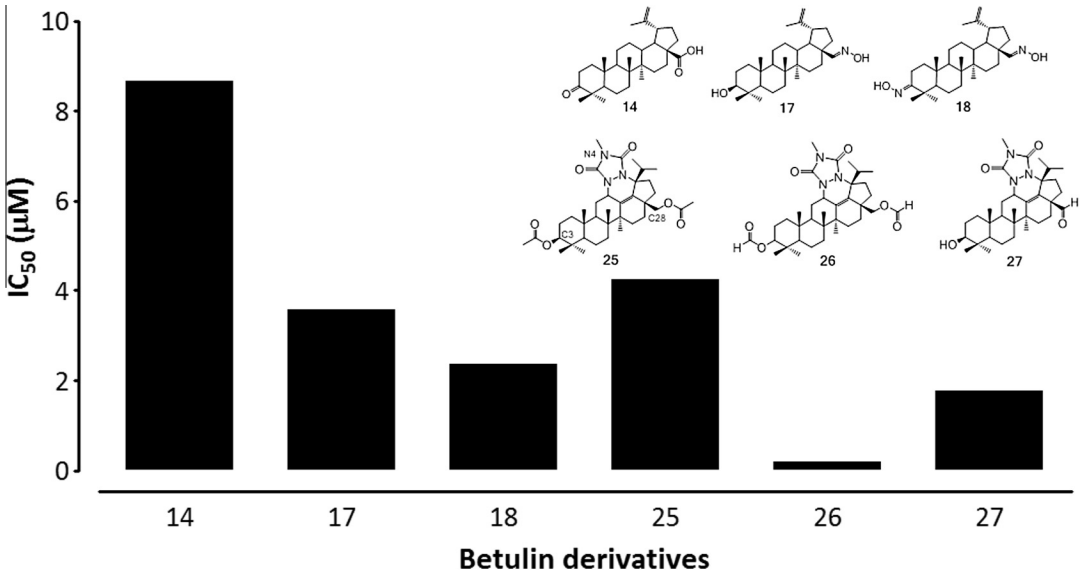


Figure 2. Chemical structures and IC_{50} of compounds **14**, **17**, **18**, and **25**–**27**.

Table 1
Comparative effect of compounds **17**, **18**, **26** and **27** against *L. braziliensis*, and macrophages J774.A1

	IC_{50} (*L. braziliensis*[1])(μM)	IC_{50} (J774.A1 [2]) (μM)	Selectivity index (IC^2_{50}/IC^1_{50})
17	4.12	>10	>2.5
18	3.35	>10	>3
26	0.25	>10	>40
27	2.06	>10	>5

4. Discussion

The work presented herein summarizes data obtained regarding betulin derivatives as leishmanicidal agents that might also impair host–parasite interaction at very low concentrations. In susceptibility tests on *L. braziliensis* the activity of 28 terpenoids was evaluated and the heterogeneous behavior of the compounds according to their structure demonstrated.

In our previous study[11] of heterocyclic betulin derivatives with antileishmanial activity, compound **9n** (i.e., compound **25** of this study, Fig. 2) with small acetyl moieties at betulin C3 and C28 hydroxyls combined with a small methyl group at N4 nitrogen of the triazolo moiety displayed the best GI_{50} value of 8.9 μM against *L. donovani*. Derivatives with bulkier substituents at C3, C28 or at N4 were less active. Additionally, C3 and C28 oxidation products of betulin improved antileishmanial activity.[10] Based on these previous results, herein we report the synthesis of three new heterocyclic derivatives (all having methyl at N4): Compound **26** with small formyl groups at C3 and C28 and two related oxidation products, that is, heterocyclic betulinic aldehyde-type compound **27** with C3 hydroxyl and C28 as an oxidation state of aldehyde as well as heterocyclic betulonic aldehyde-type compound **28** with C3 ketone carbonyl and C28 as an oxidation state of aldehyde. In the subgroup of heterocyclic triazolodione betulin derivatives, the current results correlate well with our previous results where similar compounds were assayed against *L. donovani*. The relationship

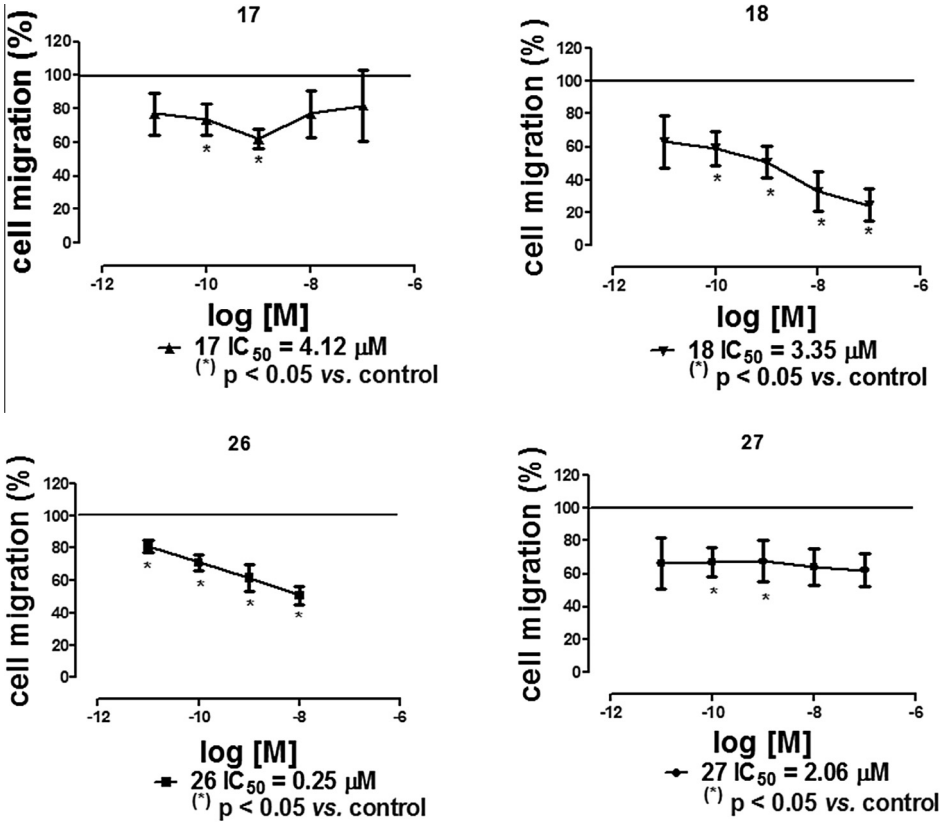


Figure 3. Effect of compounds **17**, **18**, **26**, and **27** on the rate of migration of *L. braziliensis*.

Table 2
Percentage effect of compounds **26** and **27** on the rate of infection of J774.1 macrophages by *L. braziliensis*: [1]0.5% v/v, [2]1 μM

Compound [M]	Macrophages (%)	
	With attached parasites	Infected
DMSO[1]	36.55	26.90
Amphotericin-B[2]	41.30	15.20
26 (1.00×10^{-7})	59.00	17.70
26 (1.00×10^{-8})	46.60	13.40
26 (1.00×10^{-9})	55.00	10.30
27 (1.00×10^{-7})	62.30	12.70
27 (1.00×10^{-8})	56.70	13.70
27 (1.00×10^{-9})	50.00	14.40

Table 3
Percentage of effect of compounds **26** and **27** on the average number of parasites per infected macrophage J774.A1 at three different ranges (<5, 5–10 and >10). [1]0.5% v/v, [2]1 μM

Compound [M]	Macrophages (%)		
	<5 Parasites/cell	5–10 Parasites/cell	>10 Parasites/cell
DMSO[1]	72.59	18.45	8.61
Amphotericin-B[2]	71.51	21.45	7.15
26 (1.00×10^{-7})	61.91	25.15	3.86
26 (1.00×10^{-8})	59.79	31.09	4.78
26 (1.00×10^{-9})	76.14	15.86	6.34
27 (1.00×10^{-7})	84.09	5.09	5.09
27 (1.00×10^{-8})	90.02	9.73	7.29
27 (1.00×10^{-9})	77.41	15.93	6.83

between the effect of compounds and their chemical structures was confirmed, as small groups at C3, C28 and N4 performed better than bulky groups. The new compound **28** was relatively potent, but not as active as **25**; interestingly, compounds **26** and **27** displayed improved activity compared to **25**. Derivatives **21**–**24** (Table S1), having bulkier groups at C3, C28 or at N4 showed only poor to moderate activities against *L. braziliensis*.

Moreover, the good inhibition activities of **17** with C28 oxime, **18** with C3–C28 dioxime, as well as those of betulonic acid **14** with C3 ketone carbonyl and C28 as an oxidation state of the carboxyl carbon, against *L. braziliensis* were in good correlation with the previous results of these similar compounds against *L. donovani*.

Dose–response curves were constructed for those compounds with leishmanicidal activity at concentrations lower than 10 μM that is **14**, **17**, **18**, and **25**–**27**. For compounds **17**, **18**, **26** and **27** we obtained an IC_{50} lower than (or very close to) 4 μM, suggesting that triazolodione derivatives with small C3 and C28 moieties as well as simple betulin derivatives with oxime groups at C3 or C28, favor the leishmanicidal activity. Of note, **26** IC_{50} went farther down to submicromolar values, suggesting that it could be considered as a potential hit compound for further optimization closely followed by **27** with an IC_{50} of 2 μM.

Next we ruled out the deleterious effects of the compounds on the host cell as a step in their consolidation as effective alternative agents for the control of leishmaniasis, and evaluated the action of the derivatives on the viability of the macrophage cell line J774.A1. The results suggest that terpenoids did not affect macrophage viability at the concentrations tested and that all compounds are selective against parasites over macrophages, terpenoid **26** has selectivity up to 40.

Understanding processes involved in the mutual attraction between parasite and its host cell are crucial. Chemotaxis, a phenomenon that guides cells towards or against physical or chemical gradients[28] is linked to vital events such as nutrition, breeding, infectivity, etc.[29] *Leishmania* parasites are no exception, and chemotaxis facilitates migration and successful interaction of parasite

and host cell[1,2,3] through the mutual recognition of chemical signals that determine migratory responses and infection.[5]

Herein, we studied the migratory capacity of parasites at different concentrations of the terpenoids **17**, **18**, **26** and **27**. The results demonstrated that the tested compounds exert a negative chemotactic effect, dose-dependent for **18** and **26**, and not dose-dependent for **17** and **27**. These results suggest that the betulin derivatives might decrease the probability of interaction between host cells and parasites. Of note, amphotericin-B has been suggested to affect binding of the parasite to the host cell but at higher concentrations than herein used.[30]

Finally, we explored the action of these compounds on the infectivity of *Leishmania* on their host cell. The data demonstrate that the derivatives did not significantly affect the percentage of parasites that interact and adhere to macrophages. However, the percentage of infected macrophages decreased with respect to control and in similar percentage to amphotericin-B used as a positive control of our experiments.

Moreover, compared to control conditions, compound **26** (10^{-7} M), decreased the percentage of infected macrophages containing 10 parasites or more, while **27** decreased the percentage of infected macrophages especially those containing 5–10 parasites per cell.

Of note, these effects are observed at concentrations down to three orders of magnitude for **27** and two orders of magnitude for **26** lower than their IC_{50} against promastigotes suggesting that at these low concentrations far from those exerting their leishmanicidal effect, these compounds prevent the efficient host cell–parasite interaction and installation of *Leishmania* in the host cell, thus indicating chemotactic and toxic actions might occur through different mechanisms.

In conclusion, our work suggests that cellular response analysis to identify key steps involved in the successful interaction between the parasite and the host, in their mutual recognition and migratory responses that trigger and determine the infection is a key step in the development of new types of treatment for this disease. Assays presented here indicate that the development of compounds with specific and selective directionality to tissues and cells and with multiple effects on the target cell might collaborate to minimize the selection of chemo-resistant parasites and in addition, the frequency of occurrence of adverse effects. Betulin derivatives, specifically compound **26** emerge as a viable starting point to develop new treatment options for their selectivity towards the parasite, and being safe on the host cell. Additionally, it might affect parasite–host cell interaction, as well as being leishmanicidal, in a dose-dependent manner.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.08.023.

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