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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: