Experimental Treatment of Cutaneous Leishmaniasis with Argentilactone Isolated from *Annona haematantha*

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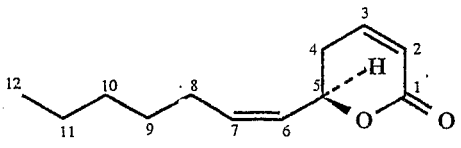
Abstract: From the hexanic extract of roots of *Annona haematantha* an α,β-unsaturated δ-lactone was isolated and identified as argentilactone. This compound exhibited *in vitro* activity against various strains of *Leishmania* ssp. at 10 μg/ml. BALB/c mice infected with *Leishmania amazonensis* were treated four weeks after infection with argentilactone by oral or subcutaneous routes for 14 days at 25 mg/kg daily. The reference drug, *N*-methylglucamine antimonate, was administered by subcutaneous injections at 100 mg/kg for 14 days. In these conditions, argentilactone showed the same efficacy as the reference drug, reducing by 96 % the parasite loads in the lesion and by 50 % the parasite burden in spleen.

Key words: *Annona haematantha*, Annonaceae, argentilactone, BALB/c mice, *Leishmania amazonensis*.

Introduction

Argentilactone, originally isolated from the rhizomes of *Aristolochia argentina* (1), is the major constituent isolated from the essential oil (2) and of the hexanic extract of *Annona haematantha* Miq., a liana growing in French Guyana. To our knowledge, this plant is not used in traditional medicine. In a preliminary biological test, argentilactone displayed activity at 10 μg/ml against *Leishmania donovani*, *L. major*, and *L. amazonensis*.

The aim of this study was to evaluate the antileishmanial activity (3) of argentilactone against *Leishmania amazonensis* when the drug was administered by oral and subcutaneous routes.



Argentilactone

Materials and Methods

General experimental procedures

Optical rotations were determined on a Schmidt-Haensch Polartronic E polarimeter. The ^{1}H- and ^{13}C-NMR spectra ($CDCl_3$) were obtained with Bruker ARX-400 and AC-200P instruments at 400 and 50 MHz, respectively. EI-MS and CI-MS (NH_4^+) were performed on a Nermag R 10-10 C spectrometer.

Plant material

Roots of *Annona haematantha* Miq. were collected by H. Jacquemin (HJ 2348) at Trois-Sauts, French Guyana. A voucher specimen has been deposited in Herbarium of ORSTOM, Cayenne.

Extraction and isolation

The dried and powdered roots (0.85 kg) were exhaustively extracted by maceration at room temperature with MeOH and the extract evaporated in vacuo to give a residue (81 g) which was extracted with a mixture of MeOH/H_2O/hexane. The hexanic fraction (4.5 g) was flash-chromatographed on a column of silica gel S (Riedel de Haen 31607, 130 g) and eluted with cyclohexane/EtOAc, 70 : 30 (1200 ml), then EtOAc (500 ml), followed by EtOAc/MeOH, 90 : 10 (400 ml). Fractions 20–25 (150 ml) were composed of the active compound (1.7 g) which was identified by its physical and spectral data as argentilactone.

Physical and spectral data

Argentilactone: yellow oil; $[\alpha]_D$: $-21°$ (*c* 1, EtOH); IR: $\nu_{max}^{CHCl_3}$ = 2932, 2859, 1421, 1244 cm^{-1}; UV: λ_{max}^{EtOH} = 216.5 nm; EI-MS: *m/z* (rel. int.) = $[M]^+$ 194 (6), 152 (10), 123 (18), 110 (20), 97 (40), 68 (100); ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 6.04 (ddd, *J* = 9.8, 1.9 Hz, H-2), 6.88 (ddd, *J* = 9.8, 4.2 Hz, H-3), 2.38 (m, H-4), 5.2 (m, *J* = 9.5, 7.8, 5.7 Hz, H-5), 5.8 (m, H-6 and H-7), 2.06 (m, H-8), 1.3 (m, H-9, H-10 and H-11), 0.9 (t, H-12); ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 164.0 (C-1), 121.1 (C-2), 145.1 (C-3), 29.7 (C-4), 73.7 (C-5), 126.3 (C-6), 135.3 (C-7), 27.5 (C-8), 28.8 (C-9), 31.2 (C-10), 22.3 (C-11), 13.8 (C-12). These data are identical to those of literature (1).

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In vivo *studies*

Mice: Female and male BALB/c mice were supplied by the IFFA-CREDO, France, and bred at the Instituto de Investigaciones en Ciencias de la Salud (IICS), Assuncion, Paraguay. Golden hamsters (*Mesocritus auratus*) were used to maintain the parasites.

Infection: *Leishmania amazonensis* IFLA/BR/67/PH8 (IFLA = strain isolated from the vector *Lutzomia flaviscutellata*, BR = Brazil, 67 = year of isolation, PH8 = laboratories references.) was used and maintained by passage every eight weeks in hamsters. BALB/c mice (n = 7) were inoculated in the right hind footpad with 1.5×10^6 amastigotes obtained from donor hamsters. The parasites were delivered in 100 μl of phosphate buffered saline (PBS). Disease progression was evaluated by the measurement of lesion diameters weekly to seven weeks.

Drug treatment: The treatments were initiated four weeks after inoculation when the infection was well established and lesions were obvious. Seven mice groups were ramdomly formed two days before administration of treatments. *N*-Methylglucamine antimonate was dissolved in 50 μl of PBS and administered to BALB/c mice in regimens of 100 mg per kg of body weight daily for 10 days by the subcutaneous route. Argentilactone was tested at a dose level of 25 mg/kg and was dissolved in 40 μl PBS; 5 μl of polysorbate (Tween 80, OSI, France) and 5 μl of dimethyl sulfoxide (DMSO). Drug was administered orally once daily for 10 days or by subcutaneous route for 10 days. The untreated group received daily 40 μl of PBS, 5 μl of Tween 80 and 5 μl of DMSO.

Effect of treatment: The animals were sacrified one week after interruption of treatment to assess parasitological loads in the infected footpad and in the spleen. Briefly, the mice were killed and the lesions of infected footpad and spleen were excised, weighed, and homogenized with a tissue glass grinder Potter in 5 ml of RPMI 1640 medium (Gibco, France) supplemented with 10% fetal calf serum, 1 ml of glutamine (Gibco, France) at 29.4 mg/l, penicillin (100 U/ml), and streptomycin (100 μg/ml). Plates were examined and the number of amastigotes per host lesion cell nucleus were counted. The number of amastigotes per lesion per nucleus × lesion weight or spleen weight in gram (10^7) is approximately equal to the total number of amastigotes per organ (4, 5). Parasite suppression was calculated from the ratio of the mean lesion amastigote counts of drug-treated mice and the mean lesion amastigote counts of untreated mice multiplied by 100 to obtain the percentage of parasite suppression.

Statistical analysis

Data are given means ± standard deviations, unless indicated otherwise. Comparison of parasite suppression in the infected footpad or in spleen of the untreated control group and drug-treated group was analyzed by two-way analysis of variance (ANOVA) and by Student's t test. Data were considered statistically significant at $P < 0.05$. Analyses were performed on a personal computer with Excel 5.0a.

Results and Discussion

The effect of treatment of BALB/c mice infected with *Leishmania amazonensis* with *N*-methylglucamine or argentilactone by the oral or subcutaneous route is presented in Table **1**. At the end of the treatment (the sixth week), we observed a decrease of the lesions size in treated mice, especially in the mice which had received argentilactone by the subcutaneous route; in this case, the decline of lesions size was up to 50%.

The treatment with reference drug or argentilactone administered by oral or subcutaneous route for 14 days showed a significant effect (Table **2**). Decreases of the lesions weight by 94% ($P < 0.05$), 71% (NS), and 95% ($P < 0.05$) and decreases in the parasite burden of the infected footpads by 95% ($P < 0.05$), 75% (NS), and 97% ($P < 0.05$) were observed. Table **2** shows the data on parasite burden in the spleen, if the spleen weights were similar, we have observed parasite dissimination in whole spleens. However, treatment with *N*-methylglucamine or with argentilactone administered by oral or subcutaneous route reduced the parasite load in the spleen by 49%, 54% ($P < 0.05$), and 48% ($P < 0.05$), respectively (see Table **2**).

In conclusion, the results obtained in this study show that subcutaneous treatment with argentilactone at 25 mg/kg for 14 days produces the same effect as treatment with the reference drug (*N*-methylglucamine antimonate). However, if the oral treatment by argentilactone seems less significant, the reduction in this case of parasites burden in the spleen appears better, with the subcutaneous route. It should be observed that the treatments with argentilactone were well tolerated and the oily form of argentilactone facilitated the administration.

Table 1 Effects of *N*-methylglucamine antimonate (= Glucantime) (100 mg per kg per day) and argentilactone administered orally at 25 mg/kg daily for 14 days and subcutaneously at 25 mg/kg daily for 14 days on the development of *L. amazonensis* (PH8) mice (Lesion size ± standard deviation) (n = 7).

Time post-infection (weeks)	Lesion size in mm — Untreated	Glucantime at 100 mg/kg	Argentilactone by oral route	Argentilactone by subcutaneous route
0	0.11 ± 0	0.18 ± 0.10	0.08 ± 0.10	0.10 ± 0.13
1	0.17 ± 0.07	0.0 ± 0.10	0.22 ± 0.10	0.22 ± 0.07
2	0.50 ± 0.17	0.43 ± 0.18	0.46 ± 0.23	0.83 ± 0.24
3	0.68 ± 0.21	0.69 ± 0.24	0.79 ± 0.22	0.91 ± 0.37
4	0.92 ± 0.31	0.92 ± 0.28	0.81 ± 0.23	1.10 ± 0.32
5	0.81 ± 0.23	0.95 ± 0.29	1.00 ± 0.36	1.47 ± 0.47
6	1.12 ± 0.39	0.73 ± 0.24	0.93 ± 0.36	0.60 ± 0.14
7	1.25 ± 0.41	0.93 ± 0.33	1.26 ± 0.43	1.17 ± 0.31

Table 2 Effects of treatments with *N*-methylglucamine antimonate by the subcutaneous route and with argentilactone by the oral or subcutaneous route on *L. amazonensis*-infected BALB/c mice (n = 7).

Parameters	Untreated mice	*N*-methylglucamine antimonate	Argentilactone	Argentilactone
Route of administration	–	subcutaneous	oral	subcutaneous
Lesion weight (g) (mean ± SD)	0.025 ± 0.032	0.0015 ± 0.0004	0.0075 ± 0.0019	0.0011 ± 0.0031
% Decrease of lesion weight	–	−94 % ($P < 0.05$)	−71 % (NS)	−95 % ($P < 0.05$)
Mean number of parasites in lesion	4.4×10^6	2.1×10^5	1.1×10^6	1.5×10^5
% Suppression of parasites burden in lesion	–	−95 % ($P < 0.05$)	−75 % (NS)	−97 % ($P < 0.05$)
Spleen weight	0.191 ± 0.026	0.201 ± 0.071	0.172 ± 0.042	0.190 ± 0.034
% Decrease of spleen weight	–	+5 %	−10 %	−1 %
Mean number of parasites in spleen	1.7×10^8	8.4×10^7	7.7×10^7	8.6×10^7
% Suppression of parasites burden in spleen	–	−49 %	−54 % ($P < 0.05$)	−48 %

Values represent the means ± standard deviation (n = 7).
$P < 0.05$ (treated versus untreated mice).

In addition, the results of this study confirmed recent observations by Almeida (6) and our group (unpublished results) about the dissemination of parasites to lymph nodes, spleen, and liver and the high susceptibility of BALB/c mice to *L. amazonensis* infection.

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