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Activity of Compounds Isolated From Chilean Lichens Against Experimental Cutaneous Leishmaniasis

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ABSTRACT. Three secondary metabolites isolated from Chilean lichens, (+) usnic acid, pannarine and 1′-chloropannarine, were tested against promastigotes forms of three strains of *Leishmania* ssp. Pannarine and 1′-chloropannarine exhibited *in vitro* activity at 50 μg/ml and (+) usnic acid at 25 μg/ml. BALB/c mice infected with *Leishmania amazonensis* were treated 4 weeks post-infection with (+) usnic acid by subcutaneous or oral routes for 15 days at 25 mg/kg or by five intralesional injections at interval of 4 days at 25 mg/kg of body weight. The reference drug, *N*-methylglucamine antimonate (Glucantime), was administered by subcutaneous injections (regimens of 28 mg of pentavalent antimony) for 15 days. The subcutaneous and oral treatments with (+) usnic did not produce any effect, but by intralesional administration we observed a significant effect that reduced by 43.34% the weight lesions and by 72.28% the parasites loads in infected footpads. *Copyright © 1997 Elsevier Science Inc.* COMP BIOCHEM PHYSIOL 116C;1:51–54, 1997.

KEY WORDS. Usnic acid, pannarine, 1′-chloropannarine, *Leishmania amazonensis*, experimental treatment, BALB/c mice

INTRODUCTION

Cutaneous leishmaniasis and mucocutaneous leishmaniasis are endemic diseases in South America, particularly in Paraguay. Leishmaniasis is initiated by inoculation of *Leishmania* species into the skin during sand fly bites. Drugs currently available for treatment of leishmaniasis are potentially toxic, are inconvenient to administer and frequently give rise to clinical resistance (2,5). The infection is classically treated with pentavalent antimony in the form of sodium stibogluconate (Pentostam) or *N*-methylglucamine antimonate (Glucantime) and with pentamidine or amphotericin B.

The Instituto de Investigaciones en Ciencias de la Salud (IICS) and ORSTOM initiated investigations to find new natural active compounds against leishmaniasis. In a preliminary screening, we selected secondary metabolites isolated from Chilean lichens, namely three phenolic compounds, (+) usnic acid, pannarine and 1′-chloropannarine (10), that displayed *in vitro* activity against promastigotes forms of three strains of *Leishmania* ssp., *L. braziliensis*, *L. amazonensis* and *L. donovani*. We describe the *in vitro* activity of the three secondary metabolites and *in vivo* leishmanicidal activity of (+) usnic acid when administered by oral, subcutaneous and intralesional routes.

MATERIALS AND METHODS

Chemicals

1′-Chloropannarine was isolated from *Erioderma leylandi* (Taylor) Müll. Arg., pannarine from *Psoroma pallidum* Nyl. and (+) usnic acid from *Protousnea malacea* (Stirt) Krog. These lichens were collected in the region of Temuco (IX Region). Fractionation and purification of these compounds were performed as previously described (4). The structures of phenolic compounds are shown in Fig. 1. *N*-Methylglucamine antimonate (Glucantime) equivalent to 0.28 mg Sb^v/ml was purchased from Rhône-Poulenc (Paris, France).

Biological Assays

Cultures of *Leishmania* ssp. were obtained from IICS (Asuncion) and identified by isoenzyme analysis. Three strains of *Leishmania* were used during these investigations: *L. braziliensis* (MHOM/BR/75/M 2903), *L. amazonensis* (IFLA/BR/67/PH8) and *L. donovani* (MHOM/IN/83/HS-70). The

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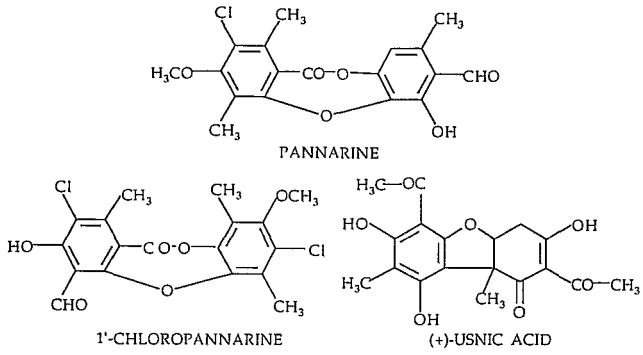

FIG. 1. Chemical structures of phenolic compounds.

maintenance, cultivation and isolation of promastigote-stage parasites were described in detail elsewhere (7).

Experimental Animals

Female and male BALB/c mice were supplied by the IFFA-CREDO, France, and bred at IICS, Asuncion, Paraguay. Golden hamsters (*Mesocritus auratus*) were used to maintain the parasites. *L. amazonensis* MHOM/IFLA/BR/67/PH8 was used and maintained by passage every 6 to 8 weeks in hamsters. BALB/c ($n = 8$) were inoculated in the right hind footpad with 2×10^6 amastigotes obtained from donor hamsters. The parasites were delivered in 100 μl of phosphate-buffered saline (PBS). Lesion development was monitored by serial measurements of footpad thickness with a dial gauge caliper (OSI, Elancourt, France). Size was expressed as the difference in thickness between the infected footpad and contralateral uninfected footpad. Measurements commenced 1 day before the inoculation of amastigotes and were continued for 8 weeks.

Drug Treatment

The treatments were initiated 4 weeks after inoculation when infection was well established and lesions were obvious. Two days before administration of treatments, the mice were randomly divided into groups of eight. *N*-Methylglucamine antimonate was dissolved in 50 μl of PBS and administered to BALB/c mice in regimen of 28 mg Sb^v/kg body weight daily for 15 days by subcutaneous route. (+) Usnic acid was tested at a dose level of 25 mg/kg and was dissolved in 40 μl PBS, 5 μl of polysorbate (Tween 80; OSI) and 5 μl of dimethylsulfoxide (DMSO). (+) Usnic acid was administered by different routes, orally twice daily for 15 days, subcutaneously once daily for 15 days or by five intralesional injections at intervals of 4 days in the infected footpad. 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 killed 1 week after cessation of treatment to assess parasitological loads in the infected footpad. Briefly, the mice were killed and the lesions of infected footpad were excised, weighed and homogenized with a tissue glass grinder (Potter) in 5 ml of RPMI 1640 medium (GIBCO, Paris, France) supplemented with 10% fetal calf serum, 1 ml of glutamine (GIBCO) 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 $\times$ lesion weight in gram (10^7) is approximately equal to the total number of amastigotes per organ (3,12). 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 means $\pm$ SD unless indicated otherwise. Comparison of parasite suppression in the infected footpad of the untreated control group and drug-treated group was analyzed by the paired Student's *t*-test. Data were considered statistically significant at $P < 0.05$ (two-tailed).

RESULTS AND DISCUSSION

After a 48-hr incubation with (+) usnic acid, 1'-chloropannarine and pannarine, the total lysis of parasites was observed at 25 and 50 μl/ml, respectively. For comparative purposes, results obtained in the presence of phenolic compounds and with the reference drugs pentamidine and ketoconazole in the culture medium are presented in Table 1.

The effects of treatments with Glucantime or with (+) usnic acid by oral, subcutaneous or intralesional routes during the course of infection of BALB/c mice infected with *L. amazonensis* are presented in Fig. 2 and Table 2. The subcutaneous treatment with antimonial drug at 28 mg/kg Sb^v for 15 days reduced significantly the lesion weight by 65.76% ($P < 0.05$) and the parasites loads by 89.55% ($P < 0.01$) vs the untreated mice. When (+) usnic acid was administered subcutaneously once daily at 25 mg/kg for 15 days or orally once or twice daily at 25 mg/kg for 15 days, we observed growth of lesions. In this case, the weight lesions increased by 72.71% ($P < 0.01$) by subcutaneous route, 46.86% ($P < 0.01$) and 17.01%, respectively, by oral route, and the parasites loads increased by 65.45% (subcutaneous route), 52.72% and 47.57% (oral administrations). Intralesional treatment with (+) usnic acid was efficient as well; in this condition, the lesion weight decreased by 43.34% and the parasite loads in the infected footpads decreased by 72.28% ($P < 0.01$) when compared with the group of untreated mice (Table 2). This specific treatment produced a slight inflammation of footpad, but generally the treatments with (+) usnic acid did not cause any obvious toxicity in the mice.

In our study, (+) usnic acid administered by intralesional route exhibited an interesting activity in BALB/c mice in-

TABLE 1. *In vitro* activity of phenolic compounds toward three strains of promastigote forms of *Leishmania* Spp.

Compounds	*L. amazonensis* (PH8) 50 μg/ml	25 μg/ml	10 μg/ml	*L. braziliensis* (2903) 50 μg/ml	25 μg/ml	10 μg/ml	*L. donovani* (HS-70) 50 μg/ml	25 μg/ml	10 μg/ml
Control	0	0	0	0	0	0	0	0	0
Pentamidine	+++	+++	+++	+++	+++	+++	+++	+++	+++
Ketoconazole	++	0		++	0		0		
(+) Usnic acid	+++	+++	++	+++	+++	++	+++	+++	+++
1′-Chloropannarine	+++	0		+++	0		+++	0	
Pannarine	+++	0		+++	0		+++	0	

0, parasites identical to control; ++, about 80–90% lysis of parasites; +++, total lysis of parasites.

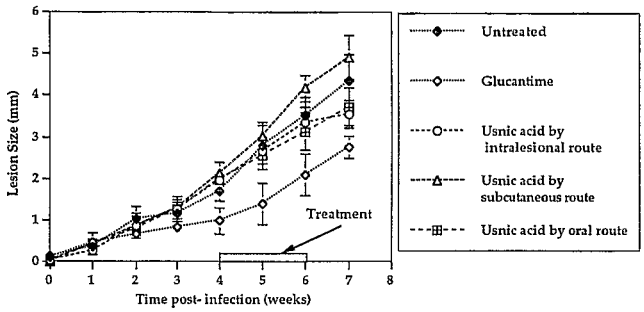

FIG. 2. Course of *L. amazonensis* infection in BALB/c mice. Groups of eight mice were either untreated, receiving PBS, or treated during the fourth and sixth weeks of infection with Glucantime at 28 mg/Sb^v/kg for 15 days or with (+) usnic acid by five intralesional injections in the infected footpad at intervals of 4 days at 25 mg/kg body weight, or with (+) usnic acid by subcutaneous route at 25 mg/kg for 15 days, or with (+) usnic acid by oral route at 25 mg/kg for 15 days. Untreated and treated mice were killed at week 7 post-infection. The mean ± SE is shown for each measurement.

fected with *L. amazonensis*, but it was less potent than antimonial compound (Glucantime). To our knowledge, this study is the first to show the leishmanicidal and antiprotozoal activity of the secondary metabolites from lichens. (+) Usnic acid and 1′-chloropannarine were described as antimicrobial compounds (6,8,9) and with antitumoral activities (1,13,14), which could explain the protozoal activity of (+) usnic acid when applied by intralesional administration. In 1995, Lauterwein *et al.* (8) indicated that (+) usnic acid had lipophilic properties; therefore, higher concentrations can be expected in ointments. These properties could explain the certain efficacy of (+) usnic acid when it was administered by intralesional route. It would be interesting to continue the evaluation of different groups of secondary metabolites—depsides, depsidones, dibenzofurane derivatives, quinones, terpenes or sterols (11)—isolated from lichens against the protozoa as *Leishmania* or *Trypanosoma cruzi*.

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TABLE 2. Effect of treatments with *N*-methylglucamine antimonate by subcutaneous route, (+) usnic acid administered by oral or intralesional or subcutaneous routes on *Leishmania amazonensis*-infected BALB/c mice

Drug (dose)	Route of administration	Lesion weight (g)	Suppression of weight lesion (%)	Suppression of parasite load in lesion (%)	Mean parasite quantitation in lesion
None (control)		0.1734 ± 0.0820*	—	—	8.95×10^6
Meglumine antimonate 28 mg Sb^v × 15	Subcutaneous	0.0593 ± 0.0126	−65.76†	−89.55‡	9.35×10^5‡
(+) Usnic acid 25 mg × 15	Subcutaneous	0.2994 ± 0.0652	+72.71‡ Inactive	+65.35‡ Inactive	1.48×10^7
(25 mg × 2) × 15	Oral	0.2546 ± 0.0584	+46.86‡ Inactive	+52.72	1.37×10^7
25 mg × 15	Oral	0.2030 ± 0.0706	+17.01	+47.57	1.32×10^7
50 mg × 5	Intralesional	0.0982 ± 0.0611	−43.34	−72.28‡ Active	2.48×10^6

*Values are means ± SD.
†$P < 0.05$ (treated mice vs control).
‡$P < 0.01$ (treated mice vs control).

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