Effect of natural naphthoquinones in BALB/c mice infected with Leishmania amazonensis and L. venezuelensis

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Abstract

Plumbagin, 3,3'-biplumbagin and 8,8'-biplumbagin are naphthoquinones isolated by activity-directed fractionation from a Bolivian plant, *Pera benensis*, used in folk medicine as treatment of cutaneous leishmaniasis caused by *Leishmania braziliensis*. BALB/c mice were infected with *L. mexicana* or *L. venezuelensis* and treated 24 h after the parasitic infection with plumbagin (5 or 2.5 mg/kg /day), 3,3'-biplumbagin, 8,8'-biplumbagin (25 mg/kg/d) or Glucantime® (200 mg/kg/d). Lesion development was the criteria employed to evaluate the inhibitory effect. The bisnaphthoquinones were less potent than Glucantime against *L. amazonensis* and *L. venezuelensis*. Plumbagin and Glucantime delayed the development of *L. amazonensis* and *L. venezuelensis*. Assays of a single local treatment on footpad infection two weeks after the parasitic inoculation with *L. amazonensis* showed that 8,8'-biplumbagin (50 mg/kg/d) was as potent as Glucantime (400 mg/kg/d).

Introduction

Cutaneous and mucocutaneous leishmaniasis are endemic diseases in the tropical subandean regions. Cutaneous leishmaniasis is popularly known as *espundia* in the area of Bolivia called Oriente by the natives. The use of medicinal plants for the specific treatment of cutaneous leishmaniasis is quite widespread, specially *Pera benensis* (Euphorbiaceae). The fresh stem barks are applied directly on the lesion.

We have previously reported the study of the chemical identification of active compounds and the leishmanial and trypanocidal activities *in vitro* of three active naphthoquinones (Fig. 1), plumbagin, 3,3'-biplumbagin and 8,8'-biplumbagin (Fournet et al., 1990). These compounds isolated by activity-directed fractionation from the stem barks and root barks of *Pera benensis*, displayed activity *in vitro* at 10 µg/ml against three strains of promastigote forms of *Leishmania* species, *L. amazonensis* (PH 8 and H-142), *L. braziliensis* (M 2903) and *L. donovani* (2682) and six strains of epimastigote forms of *Trypanosoma cruzi*. Plumbagin was also active against amastigote forms of *L. amazonensis* (PH 8) infecting the mouse peritoneal macrophages.

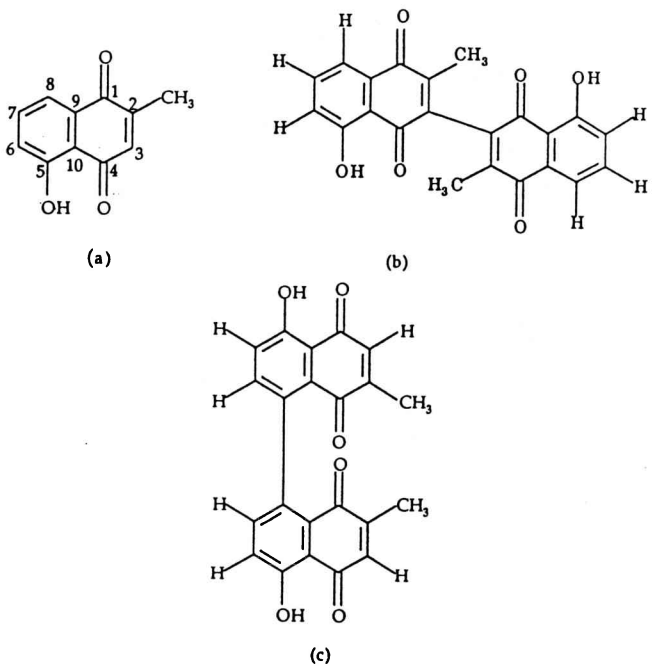

Fig. 1 Structures of (a) plumbagin; (b) 3,3'-biplumbagin; (c) 8,8'-biplumbagin

The aims of this present paper were to evaluate the activity of naphthoquinones in BALB/c mice infected with *L. amazonensis* or with *L. venezuelensis*, two species of American cutaneous leishmaniasis. We have used *L. venezuelensis* because this parasite produces for the hamster a rapid growing granuloma at the site of inoculation, containing abundant amastigotes. After a few months, we have observed necrosis on the nose and the head of the animal.

The mouse footpad infection has been used as model for these experiments (Avila et al., 1990; Coleman et al., 1989).

Materials and methods

Animals

Female or male BALB/c mice were supplied by Charles River Breeding Laboratory and then were bred in IBBA (Bolivia). Mice weighed 18–20 g and eight weeks old when bioassays were initiated.

Accepted 13 October 1992

Trop. Med. Parasitol. 43 (1992) 219–222

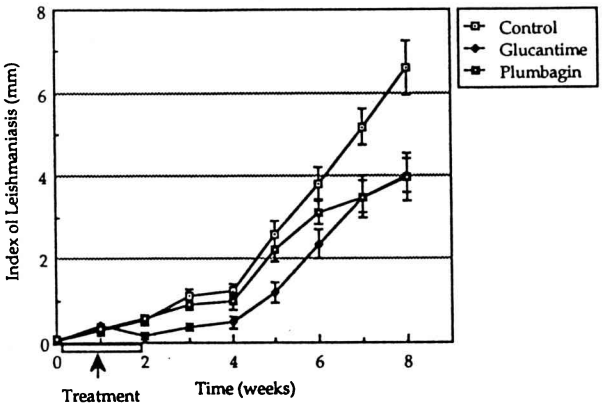

Fig. 2 Effects of plumbagin (2.5 mg/kg/d) and Glucantime (200 mg/kg/d) on the development of *L. amazonensis* (PH 8) in BALB/c mice (n = 10,-/+ S.E.M.). Treatments were given for 14 d period commencing 1 d after inoculation of *L. amazonensis* S.E.M. = −/+ Standard error of the mean

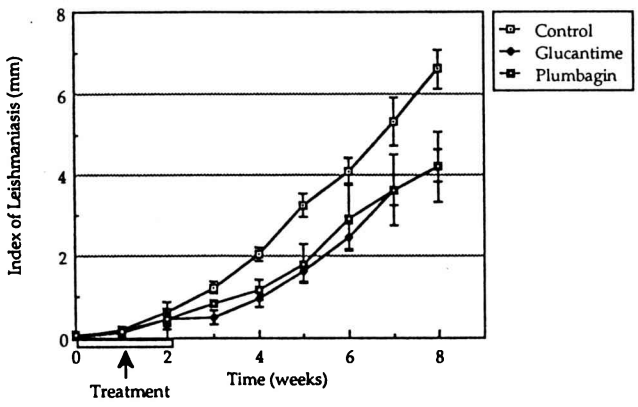

Fig. 3 Effects of plumbagin (5 mg/kg/d) and Glucantime (200 mg/kg/d) on the development of *L. venezuelensis* (H-3) in BALB/c mice (n = 6, −/+ SEM). Treatments were given for 14 d period commencing 1 d after inculation of *L. venezuelensis*.

Table 1 Effect of Glucantime (200 mg/kg/d), 3,3'-biplumbagin (25 mg/kg/d), 8,8'-biplumbagin (25 mg/kg/d) on the development of *L. amazonensis* (PH 8) in BALB/c mice (−/+SEM). Drug were given for 14 d-period commencing 1 d after inoculation of *L. amazonensis*.

Weeks post infection	Diameter of lesion* Control**	Glucantime**	3,3'-biplumbagin**	8,8'-. biplumbagin**
2	0.3 (0.15)	0.3 (0.08)	0.5 (0.13)	0.4 (0.11)
4	1.6 (0.3)	0.9 (0.23)	1.4 (0.34)	1.08 (0.28)
6	3.8 (0.6)	1.7 (0.52)	2. 7 (0.55)	2.6 (0.33)
8	6.0 (0.88)	3.23 (0.87)	4.58 (0.84)	5.05 (0.62)
9	6.9 (0.70)	3.66 (1.03)	5.05 (0.83)	5.63 (0.74)

*Average measurement (in mm) for 8 mice. ** −/+ Standard error of the mean (S.E.M.)

Leishmania strains

L. amazonensis (IFLA/BR/67/PH 8) and *L. venezuelensis* (VE/74/PM-H3) were used. The source and history of this isolate have been described by Bonfante-Garrido (1983). BALB/c mice (n = 10, n = 8 or n = 6) were infected subcutaneously in the right rear footpad with 1×10^6 amastigotes obtained from infected hamsters. The parasites were delivered in 200 µl phosphate buffered saline (PBS), with control mice receiving PBS only.

The growth of the lesion was determined weekly by measuring the diameter of both rear feet with a direct reading vernier caliper (Ref: Kroelin 10DI 00T6). The size of lesion in millimeters (Index of Leishmaniasis) was calculated by subtracting the measurements obtained for the uninfected foot from that the infected foot. Measurements started one day prior to the inoculation of amastigotes and continued for 8 or 9 weeks.

Drug treatment

Two experiments were conducted. Mice in the first experiment were treated by subcutaneous route. Glucantime was given at a dose of 200 mg/kg/d, plumbagin at 5 or 2.5 mg/kg/d, 3,3'-biplumbagin and 8,8'-biplumbagin at 25 mg/kg/d. Drug treatment started one day after the inoculation of amastigotes and continued once daily for 14 days.

In the second experiment, mice were treated directly on the infected rear footpad with a single dose 14 days after inoculation of parasites. For this experiment, mice were treated with Glucantime at 400 mg/kg/d, plumbagin at 10 mg/kg/d, 3,3'-biplumbagin and 8,8'-biplumbagin at 50 mg/kg/d.

The naphthoquinones were dissolved in 40 µl of polysorbate (Tween 80, Prolabo). For each experiment was calculated the mean and standard error of the mean (S.E.M.).

Results

Activity of naphthoquinones of Leishmania amazonensis

Separate experiments were conducted in which plumbagin was administered at different doses (7.5, 5, and 2.5 mg/kg daily) beginning 24 hr prior to infection with *L. amazonensis* (PH 8). Mice treated with 7.5 mg died within four weeks after the beginning of experiment. Figure 2 shows the combined results obtained with mice treated with 2.5 mg/kg daily of plumbagin compared to mice treated with 200 mg/kg daily of Glucantime. After eight weeks mice treated with plumbagin or with Glucantime had an average lesion size of 4 mm compared with 6.8 mm for control. We did not observe toxic effect of plumbagin at 2.5 mg/kg daily. We have obtained the same effects when plumbagin was administered at 5 mg/kg.

Table 1 presents the experiments of treatment with bis-naphthoquinones, 3,3'-biplumbagin and 8,8'-biplumbagin. These compounds were less toxic than plumbagin but less efficient at 25 mg/kg daily. After nine weeks, mice treated

Table 2 Effect of Glucantime (400 mg/kg/d), plumbagin (10 mg/kg/d), 3,3'-biplumbagin (50 mg/kg/d) and 8,8'-biplumbagin (50 mg/kg/d) on the development of *L. amazonensis* (PH 8) in BALB/c mice (−/+SEM). Drug were given on the infected rear food pad with a single treatment 14 days after the inoculation of *L. amazonensis*.

Weekspost infection	Diameter of lesion* Control**	Glucantime**	Plumbagin**	3,3'-biplumbagin	8,8' biplumbagin**
2	0.35 (0.08)	0.45 (0.05)	0.41 (0.34)	0.53 (0.21)	0.35 (0.12)
4	1.01 (0.20)	0.53 (0.23)	***	1.45 (0.38)	1.15 (0.36)
6	2.87 (0.29)	1.51 (0.37)	–	2.8 (0.64)	2.13 (0.72)
8	4.9 (0.41)	2.83 (0.58)	–	3.75 (0.45)	3.13 (1.12)

*Average measurement (in mm) for 6 mice. **−/+ Standard error of the mean (S.E.M.). *** 5 mice were dead one week after the treatment with plumbagin

Table 3 Effect of Glucantime (200 mg/kg/d), plumbagin (5 mg/kg/d), 8,8'-biplumbagin (25 mg/kg/d) on the development of *L. venezuelensis* (H-3) in BALB/c mice (−/+SEM). Drug were given for 14 d-period commencing 1 d after inoculation of *L. venezuelensis*.

Weeks post infection	Diameter of lesion* Control**	Glucantime**	Plumbagin**	8,8'-biplumbagin**
2	0.6 (0.30)	0.45 (0.19)	0.45 (0.25)	0.32 (0.08)
4	2.1 (0.19)	0.95 (0.21)	1.18 (0.26)	1.12 (0.45)
6	4.1 (0.21)	2.5 (0.34)	2.95 (0.80)	2.98 (0.85)
8	6.6 (0.51)	4.25 (0.44)	4.22 (0.85)	5.43 (0.92)

*Average measurement (in mm) for 6 mice. **−/+ Standard error of the mean (S.E.M.)

with bis-naphthoquinone had an average lesion size of 5 mm and 5.6 mm respectively compared with 3.7 mm for the mice treated with Glucantime and 6.9 mm for the untreated controls.

Table 2 shows the results of a local injection of naphthoquinones or Glucantime near the site of the lesion on the infected footpad, 14 days after the infection of mice BALB/c with *L. amazonensis*. Mice treated with plumbagin at 10 mg kg^{-1} died two weeks after the inoculation of this compound. Lesions for mice treated with 50 mg/kg of 8,8'-biplumbagin or with 400 mg/kg of Glucantime did not differ significantly after eight weeks, 3.1 mm and 2.8 mm respectively.

Activity of naphthoquinones on Leishmania vhenezuelensis

The results of the experiment with mice BALB /c infected with *L. venezuelensis* and treated 24 hr after the parasitic infection with 5 mg/kg daily of plumbagin for 14 days are presented in the Figure 3. The lesion development of mice treated with plumbagin or Glucantime were identical four weeks after the beginning of the experiment, 1.2 mm and 0.9 mm respectively. The last four weeks, the lesion size increased of 3.4 mm (Glucantime), 3 mm (plumbagin) and 5 mm (untreated controls).

Just a bis-naphthoquinone, 8-8' biplumbagin has been tested in mice infected with *L. venezuelensis* (Table 3). Lesion size of mice treated with this compound (25 mg/kg daily) and with Glucantime did not differ significantly during the first seven weeks of experiment, 3.9 mm and 3.7 mm respectively. Last week the lesion size of mice treated with 8,8'-biplumbagin or with Glucantime increased of 1.5 mm and 0.6 mm.

Discussion

The antileishmanial effect of three naphthoquinones isolated from *Pera benesis*, on mice BALB/c infected with *L. amazonensis* or with *L. venezuelensis*, is different between plumbagin and its two dimers. Our results confirm the *in vivo* activity of plumbagin against *L. amazonensis* (Croft et al., 1985) and the *in vitro* activity against promastigote and amastigote forms of *Leishmania* ssp. previously described (Fournet et al., 1990). Mice treated with plumbagin (2.5 mg/kg daily) developed an equivalent lesion size to mice treated with Glucantime. Higher concentrations of plumbagin do not increase the activity against *L. amazonensis* or *L. venezuelensis*, but produce toxic effects for mice. We have observed local necrosis in site of the treatment and weight loss during the first five weeks.

The both dimers of plumbagin, 3,3'-biplumbagin and 8,8'-biplumbagin, are less toxic but less active than plumbagin. Only 8,8'-biplumbagin has showed an equivalent activity as Glucantime when administered locally in the infected rear footpad.

This study has demonstrated that stem barks of *P. benensis* are efficient to cure the lesions of cutaneous leishmaniasis caused by the protozoan *L. braziliensis*. This antileishmanial activity is due to presence of high concentration of naphthoquinones, specialy plumbagin. Naphthoquinones affect *Leishmania* ssp. by generating free oxygen radicals (Docampo et al., 1978; Neal and Croft, 1984) within parasites which are defective in protective mechanisms against oxygen radicals, particularly catalase (Goijman and Stoppani, 1985).

The results obtained with plumbagin are similar to those observed (Croft et al., 1985) using mice BALB/c

infected with *L. amazonensis* (LV/78) or *L. donovani* (LV9). A bis-naphthoquinone isolated from the Indian plant, diospyrine, a dimer of 7-methyl-juglone is also active *in vitro* against *L. donovani* (Hazra et al., 1987). Several authors have reported an antiprotozoal activity of naphthoquinones (Callahan et al., 1988; Pinto et al., 1987; Wright and Phillipson, 1990), particularly of lapachol and β-lapachone isolated from *Tabebuia rosea* (Bignoniaceae) against *Plasmodium falciparum* (Carvalho et al., 1988) and against *Trypanosoma cruzi* (Goncalves et al., 1980), and a derived of lapachone, lapinone (Hudson et al., 1985) against *Plasmodium vivax*. Recently synthetic naphthoquinones (566C80) have been described as active against *Pneumocystis carinii* (Wellcome Foundation, 1990). Several authors have described the activity of naphthoquinones against skin diseases, plumbagin (Gujar, 1990) and 2-hydroxy-1,4-naphthoquinones for prevention of dermatitis on the scalps (Tsucha and Yutaka, 1990).

In conclusion, the results of this study show that treatment with a topical application directly on the lesion of leishmaniasis of stem barks of *Pera benensis* may be effective against leishmaniasis. It could be possible to propose an effective ointment prepared locally with a low concentration of plumbagin or an other derived of this napthoquinone less toxic as 8,8'-biplumbagin. These formulations would be developed in endemic regions of cutaneous and mucocutaneous leishmaniasis, in particular in areas of colonization of Bolivia when occure the lack of usual drugs as pentavalent antimonials.

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