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In vitro Activity and Mutagenicity of Bisbenzylisoquinolines and Quinones Against *Trypanosoma cruzi* Trypomastigotes

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The accidental transmission of Chagas' disease by donor blood is recognized as a serious problem in the Latin America. This paper describes the screening of natural products as possible new chemoprophylactic additives in blood banks. Ten plant-derived alkaloids, three terpenes, three quinones and 14 crude plant extracts were tested against bloodstream forms of *Trypanosoma cruzi* Y strain *in vitro* at 4 °C at a concentration of 250 μg/mL, using gentian violet as the baseline drug. The bisbenzylisoquinoline alkaloids, cocsuline, daphnandrine, daphnoline, isochondodendrine, gyrocarpine, limacine and pheanthine and the naphthoquinone, plumbagin completely lysed the trypomastigote forms of *T. cruzi* at a concentration of 250 μg/mL, this activity was verified by the subculture of the treated medium during 4 months. The active alkaloids, pheanthine, daphnoline and limacine were evaluated for mutagenicity by the sister chromatid exchange assay (SCE) in peripheral lymphocytes. Daphnoline and pheanthine elicited no significant increase of the SCE up to 50 μg/mL, while limacine significantly increased the SCE values at a concentration of 25 μg/mL, and 50 μg/mL.

Keywords: Trypanosoma cruzi; trypomastigote; bisbenzylisoquinoline alkaloids; mutagenicity; naphthoquinones; terpenes.

INTRODUCTION

Chagas' disease is a widespread infection in Central and South America. It is caused by the protozoan *Trypanosoma cruzi* and is naturally transmitted by Reduviidae bugs (Garcia-Zapata and Marsden, 1986). Blood transfusion is the second most important mechanism of transmission in both endemic and non-endemic areas. This fact is of epidemiological importance due to the migration of infected individuals from endemic rural environments to urban areas. It has been estimated that about 20 000 blood samples could be transmitting the disease annually by blood transfusion in Latin America (Schmuñis, 1985). Another situation relates to hospitals and small health centres in endemic regions, which lack facilities to store blood, thus practising immediate transfusion (Croft *et al.*, 1988; Docampo *et al.*, 1988). The *in vitro* trypanocidal effect of the triphenylmethane dye gentian violet (crystal violet) was first reported by Nussenzweig *et al.* in 1953. Gentian violet is added to blood samples at a concentration of 250 μg/mL and treated blood is stored 24 h before use. The main disadvantages of this treatment are the time required for drug action before transfusion and the colouring of the blood (Docampo *et al.*, 1988). Gentian violet shows no serious toxic effects at recommended concentrations (Rezende *et al.*, 1965). However, a potential carcinogenicity in rodents (Littelfield *et al.*, 1985) and a low mutagenicity have been reported (Docampo and Moreno, 1985; Thomas and MacPhee, 1984). Furthermore, the use of gentian violet is not easily accepted by patients and medical doctors (Letelier *et al.*, 1990).

Several plant derived and synthetic compounds as well as crude plant extracts have been evaluated as trypanocidal agents against epimastigote and trypomastigote forms of *Trypanosoma cruzi in vitro* (Aldunate *et al.*, 1986; Cavin *et al.*, 1987; Croft *et al.*, 1988; Fournet *et al.*, 1988b; Chiari *et al.*, 1991). In the present work, the trypanocidal effect of crude plant extracts and plant-derived compounds, comprising alkaloids, terpenes and naphthoquinones was assessed on the bloodstream forms of *T. cruzi*. The active products were also evaluated for mutagenicity by induction of sister chromatid exchange (SCE) in peripheral blood lymphocyte culture.

MATERIAL AND METHODS

Plant material. The plants were collected in Bolivia between 1985 and 1988. Herbarium specimens were submitted for identification by botanists in the Herbariums in Bolivia, United States and Europe. Voucher specimens were deposited in the National Herbarium of Bolivia in La Paz. Crude extracts were prepared from 12 plants belonging to the botanical families Annonaceae, Araliaceae, Asteraceae, Euphorbiaceae, Lauraceae, Sapindaceae and Ulmaceae. Pure compounds were isolated from natural sources and identified by spectral data (NMR, mass, IR, UV).

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Parasites. *Trypanosoma cruzi* Y strain infected albino mice were used 7 days after infection. Blood was obtained by cardiac puncture using 3.8% sodium citrate as anticoagulant in a 7:3 blood:anticoagulant ratio. The parasitaemia ranged from 1×10^5 to 5×10^5 parasites per mL. Plant extracts were dissolved in cold DMSO (dimethy sulphoxide) to a final concentration of 250 μg/mL. Aliquots of 10 μL were mixed with 100 μL of infected blood, using a microtitre plate in triplicate. DMSO plus infected blood and infected blood alone, and as baseline activity, infected blood with gentian violet at 250 μg/mL were used as controls. Different concentrations of parasites (1×10^5 and 1×10^6 parasites per mL) and extracts (4, 20, 40, 100 and 250 μg/mL) were used. The plates were shaken for 10 min at room temperature and kept at 4 °C for 24 h. Each solution was microscopically observed at 400×, placing a 5 μL sample on a slide and covering it with a 22 × 22 mm overglass for parasite counting (Schempler, 1978). Samples negative to *T. cruzi* by microscopy were cultivated in liver infusion tryptose medium (LIT) and observed for 4 months to detect epimastigote forms (Chiari and Camargo, 1984). Lethal concentrations (LC_{50} and LC_{90}) were calculated for each extract with a log-probit and means. Variance and standard deviation were calculated for each extract concentration.

Induction of sister chromatid exchange (SCE). Human lymphocytes were obtained from healthy donors and cultures were prepared using Hungerford's modified method (Rossner *et al.*, 1987). One half ml of heparinized blood was incubated in 5 mL of RPMI 1640 medium supplemented with 10% bovine serum, 0.1 mL phytohaemagglutinin and 7.5% sodium bicarbonate at 37 °C for 72 h. Tested materials were dissolved in DMSO and added to the culture. 5-bromodeoxyuridine (BrdU) was added to each culture to a final concentration of 5 μg/mL. Incubation was performed in the dark to avoid SCE induced by photolysis of BrdU-substituted DNA. After two rounds of replication, demecolcine (0.02 μg/mL) was added to each culture 2 h before harvesting. Cells were treated with a hypotonic solution, fixed with methanol/acetic acid and dropped on clean chilled glass slides. Differential sister chromatid staining was performed as described by Perry and Wolff (1974). In each sample, 20 metaphases showing differences in chromatid staining were observed and photographed. Terminal SCE were scored as 2. SCE in the centromeric region were not scored because they were indistinguishable from the twisting of sister chromatids.

Table 1. Activity of crude plant extracts towards trypomastigote forms of *Trypanosoma cruzi in vitro*

Voucher[a]	Botanical name	Part[b] and extract[c]	Lysis at 250 μg/mL (%)
	ANNONACEAE		
A.F. 481	*Oxandra espintana* (Spruce) Baill.	SB, PE	21
	ARALIACEAE		
A.F. 760	*Oreopanax* sp.	L, Ch	29
	ASTERACEAE		
A.F. 631	*Jungia polita* Griseb.	AP, Ch	31
A.F. 616	*Munnozia fournetii* H. Robinson	L, EtOH	16
A.F. 434	*Munnozia maronii* André	L. PE	29
A.F. 768	*Ophryosporus piquerioides* (DC) Benth	AP, EA	29
A.F. 675	*Senecio hieronymii* Griseb.	L, EA	16
	EUPHORBIACEAE		
A.F. 846	*Pera benensis* Rusby	SB, Ch	31
	LAURACEAE		
A.F. 856	*Aniba* sp.	SB, Ch	29
		SB, PE	29
A.F. 786	*Aniba canelilla* H. B. K.	SB, PE	22
		RB, PE	35
	SAPINDACEAE		
A.F. 780	*Serjania tenuifolia* Radlk.	L, EtOH	17
	ULMACEAE		
A.F. 884	*Ampelocera edentula* Kuhlm.	SB, Qui.	24
	Gentian violet		100

[a] Voucher: Voucher number refers to A. Fournet's collection.
[b] Part: AP, aerial part; B, bark; L, leaf; S, stem.
[c] Extract: Ch, chloroform; EA, ethyl acetate; EtOH, ethanol; PE, petroleum ether.

Table 2. Effect of selected plant-derived alkaloids, quinones and terpenes on the trypomastigote forms of *Trypanosoma cruzi in vitro*

Compounds	Lysis at 250 μg/mL (%)	Reference
Gentian violet	100	
Alkaloids		
Cocsoline	100	Lavault *et al.* 1987
Cycleanine	54	Hocquemiller *et al.*, 1984
Daphnandrine	100	Lavault *et al.*, 1987
Daphnoline	100	Lavault *et al.*, 1987
Gyrocarpine	100	Chalandre *et al.*, 1986
Isochondodendrine	100	Lavault *et al.*, 1985
Limacine	100	Lavault *et al.*, 1986
Obaberine	37	Weber *et al.*, 1988
Pheanthine	100	Chalandre *et al.*, 1986
Quinones		
Plumbagin	100	Fournet *et al.*, 1992
3,3′-biplumbagin	41	Fournet *et al.*, 1992
8,8′-biplumbagin	39	Fournet *et al.*, 1992
Terpenes		
Dehydrozaluzanin C	31	Fournet *et al.*, 1993
Espintanol	2	Hocquemiller *et al.*, 1991
Isoespintanol	8	Hocquemiller *et al.*, 1991

RESULTS

From the crude extracts, *Oreopanax* sp., *Jungia polita, Munnozia maronii, Ophryosporus piqueriodes, Pera benensis* and *Aniba* sp. (Table 1) showed a third of the activity of the standard trypanocide gentian violet and are worthy of further studies. The naphthoquinone plumbagin showed 100% lysis while the dimeric derivative, 3,3′-biplumbagin and 8,8′-biplumbagin were half as active as the corresponding monomer. The terpenoids dehydrozaluzanin C, espintanol and isoespintanol proved to be weakly active in our assay conditions.

Table 3. Determination of lethal concentration of compounds tested *in vitro* against trypomastigotes of *T. cruzi*

Compound	LC_{50} (μg/mL)	LC_{90} (μg/mL)
Pheanthine	40	175
Limacine	35	180
Daphnoline	8	50
Coesoline	–	4

Table 4. Frequency of sister chromatid exchanges (SCE) in peripheral lymphocytes induced by active compounds at different concentrations

Compound	Concentration (μg/mL)					
	100	50	25	10	5	DMSO 1%
Pheanthine	11.3±2.6[a]	8.1±2.5[a]	6.4±1.9	5.3±1.7	5.3±1.7	5.9±1.4
Limacine	ND	23.6±4.7[a]	19.5±1.8[a]	8.8±2.3[a]	8.8±2.3[a]	5.9±1.5
Daphnoline		9.1±2.5[a]	7.3±2.4[a]	7.1±2.8	7.1±2.8	5.8±2.3

Values are presented as mean±SD
[a]Significantly different from control ($p<0.001$)
ND: not done

The bisbenzylisoquinoline alkaloids, cocsoline, daphnandrine, daphnoline, isochondodendrine, gyrocarpine, limacine and pheanthine completely lysed the bloodstream forms of *T. cruzi* (Table 2) at a concentration of 250 μg/mL thus being comparable in activity to gentian violet. The means and lytic concentrations (LC_{50} and LC_{90}) were determined for four of the ten alkaloids (Table 3).

Daphnoline and coscoline showed an interesting activity when compared with gentian violet. Furthermore, daphnoline was not cytotoxic at concentrations below 10 μg/mL. When it was tested at a concentration at 250 μg/mL under low parasitaemia, no parasites were detected in our conditions. Pheanthine and limacine showed LC_{50} and LC_{90} fivefold higher than daphnoline. The mutagenicity of pheanthine, limacine and daphnoline was also assessed (Table 4). Pheanthine and daphnoline elicited no significant increase of the SCE up to 50 μg/mL, while limacine significantly increased the SCE values at 25 and 50 μg/mL. The spontaneous frequency of aberrant cells in negative controls was in the range of 0%–5.9%.

DISCUSSION

All the crude extracts and the pure compounds presented an inhibitory effect on the promastigote forms of *Leishmania* and the epimastigote forms of *Trypanosmoa cruzi in vitro* (Fournet, 1991). In this study no crude extract was found active against the blood forms of *Tryponosoma cruzi in vitro*. Seven bisbenzylisoquinoline alkaloids of nine tested showed an activity on the trypomastigote forms of *T. cruzi*, particularly cocsoline and daphnoline which showed no mutagenic effects. The same compounds previously presented an inhibitory activity *in vitro* against different strains of promastigote forms of *Leishmania* ssp. and epimastigote forms of *T. cruzi* at a concentration of 10 μg/mL (Fournet *et al.*, 1988a, 1988b). Recent publications have demonstrated the antiprotozoal properties of the bisbenzylisoquinonines, *in vitro* against *Trypanosoma brucei brucei*, *Plasmodium falciparum* and *P. berghei berghei* (Schiff, 1991). In our study, no structure–activity relationship with the bisbenzylisoquinoline alkaloids was observed.

The only active naphthoquinone, plumbagin is an antiprotozoal compound with activity against *L. amazonensis* and *L. donovani in vitro* and *in vivo* (Croft *et al.*, 1985; Fournet *et al.*, 1992) and antibacterial and antifungal activities (Gujar, 1990). Furthermore, by topical application it also shows a significant suppression of skin lesions (Fournet, 1991; Wright and Phillipson, 1990). The result demonstrates again that compounds known to generate free radicals, for example plumbagin, are effective trypanocidal compounds *in vitro*.

In conclusion, it would be interesting to complete this study by testing these alkaloids and other bisbenzylisoquinoline alkaloids against other strains of *T. cruzi*, and also their effectiveness at 37 °C or 25 °C, the same conditions to those in the small health centres of rural Latin America. Furthermore, the compounds tested have the disadvantage of there being no product licences for use in human medicine and it is not easy to isolate the bisbenzylisoquinoline alkaloids by natural or synthetic route.

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