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In Vitro and in Vivo Assessment of the Antimalarial Activity of Sergeolide

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Abstract: The antimalarial activity of sergeolide (a quassinoid from *Picrolemma pseudocoffea*) was investigated both, *in vitro* on *Plasmodium falciparum* cultures and *in vivo* through a classical test of schizontocidal action against *Plasmodium berghei* in mice. Sergeolide showed a very strong antiplasmodial activity *in vitro* as well as *in vivo*. Low concentrations (0.006 µg/ml) were able to fully inhibit the *in vitro* growth of chloroquine-sensitive and resistant strains of *P. falciparum*. Small amounts (0.26 mg/kg/day) markedly reduced the virulence of experimentally induced *P. berghei* infection in mice. However, sergeolide, because of its high toxicity (LD 50: 1.8 mg/kg), does not seem, in its present form to be useful for malaria curative treatment.

Introduction

Prophylactic and curative malaria treatment still depend on the use of classical drugs (chloroquine and its derivatives, pyrimethamine and sulphonamides). However, in numerous part of the world, resistance to these common antimalarial drugs has become so widespread and intense that the development of new compounds for malaria therapy is a real emergency (1).

A recent study, interestingly, has shown that certain quassinoids (bitter principles of the plant family *Simaroubaceae*) (2) were capable of inhibiting the *in vitro* growth of chloroquine-resistant strain of *P. falciparum* (3). This latter work unfortunately, did not connect the *in vitro* antimalarial activity of quassinoids with the presence or absence of antimalarial effects *in vivo* in experimental animal models.

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Our communication is concerned with a study carried out to determine the antimalarial activity *in vitro* and *in vivo* of a new quassinoid, sergeolide, recently isolated from the French Guinanan *Simaroubaceae*: *Picrolemma pseudocoffea*. Well known by the indigenous population for its antimalarial properties, *Picrolemma pseudocoffea* has for a long time been employed in traditional medicine for malaria therapy (4).

Materials and Methods

Drugs

The extraction and purification of the sergeolide from the dried ground roots of *Picrolemma pseudocoffea* were carried out using a method already described (5). The compound in pure crystallized state was prepared according to the method established by Trager et al. (3). Briefly, sergeolide was dissolved in 95 % ethanol at a concentration of 3 mg/ml.

For the *in vitro* study, a stock solution was prepared by diluting the ethanol solution in the proportion of 1 : 10 with RPMI culture medium (without bicarbonate). The stock solution was sterilized through a 0.22 µm filter (Schleicher and Sch ull), then, in order to obtain final concentrations of sergeolide ranging from 0.002 to 0.5 µg/ml, diluted aseptically prior use in complete RPMI medium.

To investigate *in vivo* the acute toxicity and the antimalarial activity of sergeolide, the original ethanol solution was diluted with normal saline to obtain solutions ranging from 0.0015 to 1.2 mg of sergeolide per ml.

Chloroquine solutions were prepared in the same way from chloroquine disulfate but, in this case, the initial solubilization was directly performed in saline (3 mg of chloroquine per ml of solution).

Control solutions, containing equal amounts of ethanol to those introduced with the sergeolide, were also prepared either from a stock solution of 10 % ethanol in RPMI culture medium for the *in vitro* study (range of dilutions 1 : 1,500,000–1 : 1,000) or directly from 95 % ethanol for the *in vivo* study (range of dilutions 1 : 200–1 : 2.5).

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In vitro measurement of the antimalarial activity

Parasite strains: four strains of *P. falciparum* were used throughout this work.

Originally obtained from R. J. M. Wilson (London, U. K.), the chloroquine-sensitive strain Ouganda Palo Alto (FUP strain) has since been fully adapted to the squirrel monkey (*Saimiri sciureus*), without losing its infecting potential for human red blood cells (6). This strain routinely maintained on splenectomized animals, was isolated from a highly infected *Saimiri* (30% parasitemia), then cultivated in human A⁺ erythrocytes for more than a month prior to be used for this study.

Three strains of Brazilian origin, namely 95/83, 96/83 and 97/83, kindly provided by V. E. Do Rosario (Belem, Brazil), were brought to our laboratory as parasitized blood, directly isolated from patients. They have all since been adapted to continuous culture in human red blood cells. All three are chloroquine-resistant using Rieckmann's method (7) (Rosario, personal communication).

Culture procedure: The cultures were carried out in human A⁺ red blood cells, according to Trager and Jensen's methodology (8). The culture medium used throughout was RPMI 1640 supplemented with 25 mM HEPES and 10% human A⁺ serum. The complete culture medium with different concentrations or dilutions of the compound to be test or without drugs (for the controls), was distributed into 16 mm wells of a 24 well plate (0.5 ml/well). Each well the received 50 µl of a parasitized suspension, obtained by mixing red blood cells suspended in RPMI with enough infected erythrocytes to give an initial parasitemia ranging from 0.35 to 0.70%. Parasitized red cells were shaken gently with a vortex mixer, before and during their distribution into the wells. The plates were then incubated at 37°C under a continuous flow of a humidified gas mixture containing 2% O₂, 3% CO₂, and 95% N₂. Giemsa stained thin blood films were prepared at times 0, 24, and 48 hours.

Measurement of the acute toxicity of sergeolide

The lethal dose 50 (LD 50) of sergeolide was determined from the graph obtained by plotting the doses (logarithmic scale) against the percentages of mortality (probit scale).

0.5 ml volumes of increasing doses of sergeolide (0.3, 2.2, 6.6 and 19.8 mg/kg) were randomly administrated by subcutaneous injections to groups of 10 Swiss mice (bred at the "Institut Pasteur de la Guyane Française", average weight: 30 ± 2 g). The mortality was noted after 72 hours.

In order to determine the ethanol toxicity, control groups of 10 mice were treated with different dilutions of ethanol (1:167, 1:23, 1:7.5, 1:2.5) corresponding to those used with the different doses of sergeolide.

In vivo measurement of the antimalarial activity

The antimalarial activity of sergeolide was determined and compared with that of chloroquine by a classical 4 day suppressive test against *P. berghei* in mice (9). The *P. berghei* strain used was the NK 65 strain, obtained from J. P. Vandenberg (New York, U.S.A.).

80 male Swiss mice (average weight: 29 ± 1 g) were injected intraperitoneally with 10⁷ parasitized cells in 0.2 ml normal saline. The day of infection was numbered day 0 (DO) and the following, D + 1, D + 2, etc. . . .

Groups of 10 mice were randomly injected subcutaneously from DO to D + 3 with different doses of sergeolide (0.26, 0.09 and 0.026 mg/kg/day), chloroquine (1.3, 0.4 and 0.13 mg/kg/day), or ethanol (1:200 dilution) in 0.5 ml normal saline. The suppressive effects of the different compounds was estimated at D + 4 on Giemsa stained thin blood films, in comparison with a group of 10 mice treated with normal saline.

Results

Antimalarial activity in vitro

The *P. falciparum* growth is strongly inhibited by sergeolide. As shown in Table I, 50% inhibition could be observed, in the case of the chloroquine-sensitive FUP strain, for very weak concentrations (0.002 and 0.006 µg/ml), higher concentrations resulting in almost complete inhibition. However, with regard

Table I. Effects of sergeolide *in vitro* on the chloroquine sensitive strain FUP of *Plasmodium falciparum*

Drugs*	Concentrations µm/ml	Parasitemia after 24 h culture**			Parasitemia after 48 h culture**				Inhibitions*** %	
		Parasitemia %	Differential counts			Parasitemia %	Differential counts			
			R	T	S		R	T		S
None	0	0.9 ± 0.1	86	10	4	1.4 ± 0.1	68	13	9	0
	0.5	0.16 ± 0.02	100	0	0	0.09 ± 0.08	100	0	0	94
	0.17	0.09 ± 0.02	83	11	6	0.10 ± 0.01	100	0	0	93
	0.05	0.12 ± 0.02	88	6	6	0.08 ± 0.02	100	0	0	94
SG	0.018	0.33 ± 0.13	93	7	0	0.23 ± 0.05	100	0	0	84
	0.006	0.4 ± 0.1	96	4	0	0.63 ± 0.09	100	0	0	55
	0.002	0.63 ± 0.07	93	5	2	0.8 ± 0.2	66	27	7	43
	0.5	0.21 ± 0.05	63	37	0	0.07 ± 0.01	78	22	0	95
CL	0.17	0.13 ± 0.02	84	16	0	0.10 ± 0.01	82	18	0	93
	0.05	0.29 ± 0.02	95	3	2	0.23 ± 0.04	79	21	0	84
	0.018	0.51 ± 0.03	94	5	1	0.35 ± 0.03	81	17	2	75
	0.006	0.53 ± 0.05	88	6	6	1.2 ± 0.1	67	14	19	14
	0.002	0.63 ± 0.09	88	8	4	1.2 ± 0.1	67	11	22	14
	1:6,000	0.72 ± 0.02	89	6	5	1.3 ± 0.2	68	18	14	7
	1:18,000	0.7 ± 0.1	86	9	5	1.4 ± 0.2	56	17	27	0
	1:54,000	0.81 ± 0.07	84	12	4	1.3 ± 0.1	59	17	24	7
EtOH	1:162,000	0.58 ± 0.17	90	7	3	1.49 ± 0.02	50	25	25	0
	1:486,000	0.71 ± 0.04	80	13	7	1.2 ± 0.3	63	11	26	14
	1:1460,000	0.5 ± 0.2	84	13	3	1.3 ± 0.2	60	15	25	7

* - SG, sergeolide; CL, chloroquine; EtOH, ethanol.

** - Percentages of parasitemia are the mean values ± SD calculated from triplicates experiments. At time "0" the count was; 0.35 ± 0.03% - R (ring), 71; T (trophozoite), 0; S (schizonte), 29.

*** - Percentages of inhibition were calculated at time "48" against control culture samples without drugs.

to chloroquine, its antiparasitodal activity becomes significant from threshold concentration of $0.018 \mu\text{g/ml}$. The culture samples containing equal of ethanol as were introduced with segeolide reacted in the same way as control culture samples without drugs. Differential parasite counts revealed that segeolide, like chloroquine, is simultaneously effective against all asexual blood stages.

Concerning the three chloroquine-resistant strains *P. falciparum* (including the 97/83 isolate which showed *in vivo* a resistance to the association quine/fansidar) their *in vitro* growth were equally inhibited by segeolide (Fig. 1). The drug is highly active at concentrations $\geq 0.006 \mu\text{g/ml}$, whereas an identical inhibitory effect can only be obtained with chloroquine at a 100 fold higher concentrations ($0.5 \mu\text{g/ml}$).

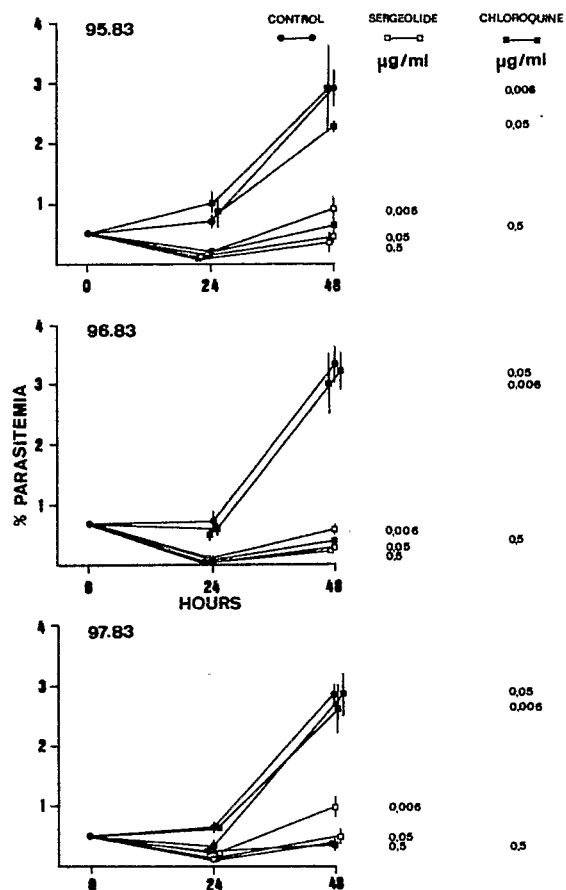


Fig. 1. Effects of segeolide *in vitro* on three chloroquine-resistant strains of *Plasmodium falciparum*. Each point is the mean value \pm SD calculated from triplicate experiments.

Acute toxicity of segeolide

For the calculation of the LD 50, we did not take into account the results from the group of mice injected with 19.8 mg/kg , because in the control group treated with ethanol at a 1 : 2.5 dilution, two animals died. The quantity of ethanol introduced for this dilution ($\approx 5 \text{ g/kg}$) corresponds in fact, to the LD 50 of ethanol already described (10). No deaths were observed in the group of mice treated with smaller doses of ethanol. The results of our test fixed the LD 50 of segeolide at 1.8 mg/kg .

Antimalarial activity *in vivo*

We determined the minimum effective dose of segeolide able to reduce by half the virulence of *P. berghei* infection experimentally induced in mice (ED 50).

Doses of segeolide were chosen according to the LD 50 previously established. In order to check the reliability and evolution of the experiment, a dose-response curve was also plotted for a known drug, chloroquine.

From Figure 2 the ED 50 are:

- segeolide: approximately 0.2 mg/kg/day (range of value in dose, 0.08 to 0.4)
- chloroquine: approximately 1 mg/kg/day (range of value in dose, 0.16 to 4).

The group of 10 mice treated with 0.5 ml of a 1 : 200 ethanol dilution, which corresponds to the quantity of ethanol introduced with the segeolide at its highest dose (0.26 mg/kg/day) developed at D + 4 an average parasitemia identical to that of the control group treated with normal saline.

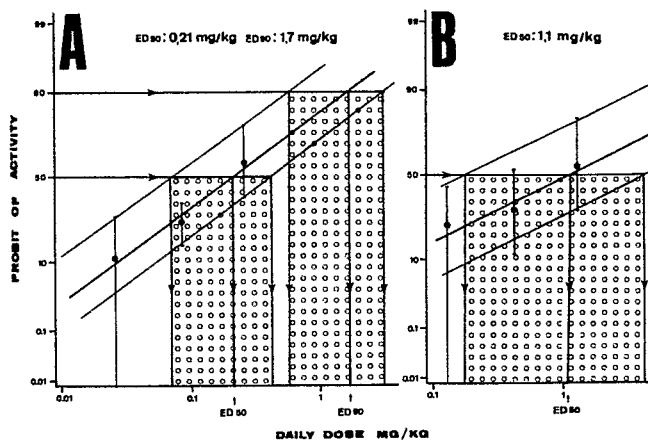


Fig. 2. Effects of segeolide and chloroquine *in vivo* on the NK 65 strain of *Plasmodium berghei* in mice.

A, segeolide; B, chloroquine.

Daily doses of drugs are plotted on a logarithmic scale.

Discussion

Earlier work showed that the antimalarial activity of quassinoids runs in parallel to their antileukemic properties (3).

Considering the original structure of segeolide (first known example of quassinoid with a butenolide function attached to the ring A), (Fig. 3) and its highly inhibitory action against mouse leukemia P 388 cell line, it seemed of interest to check the antimalarial activity of this new molecule, *in vitro* and *in vivo*.

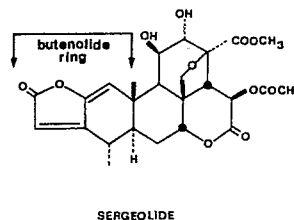


Fig. 3. Structural formula of segeolide.

As suspected from the preliminary tests on its toxicity (5), sergeolide possesses a high antimalarial activity. *In vitro* by a 48 hour culture method, it showed a significant inhibitory effect against *P. falciparum* at a concentration of 0.006 µg/ml (Fig. 1) and even at 0.002 µg/ml (Table I). A comparable inhibitory action can only be obtained with chloroquine at a concentration 3 fold higher in the case of a chloroquine-sensitive strain (Table I) and 100 fold higher for chloroquine-resistant strains (Fig. 1). It is of interest to note that the strain 97/83 having shown *in vivo* a resistance to the association quinine/fansidar, is identically sensitive to the antiparasitical action of sergeolide.

Sergeolide, although very active *in vivo* (ED 50 ≈ 0.2 mg/kg/day) at lower concentrations than that chloroquine (ED 50 ≈ 1 mg/kg/day), presents unfortunately, little interest in the curative treatment of rodent malaria (ED 90 ≈ 1.7 mg/kg/day), because of its high toxicity (LD 50: 1.8 mg/kg).

Our results argue that the antimalarial activity of *Picrolemma pseudocoffea* could be imputed to the presence of sergeolide.

Isobrucine B, another highly toxic quassinoid (LD 50 approximately 5 mg/kg) isolated from this French, Guianan *Simaroubaceae*, seems, on account of its 5 fold lesser antimalarial activity *in vitro* (results not shown here), take only a moderate part in the antimalarial plant activity.

If, as we suggest, sergeolide is the principal antimalarial compound of *Picrolemma pseudocoffea*, it remains, taking its high toxicity into account, to clarify the effective utilization of *Picrolemma pseudocoffea* in traditional medicine. It will be relevant to collect more information concerning the direction for use of the plant in malaria therapy (preparation, doses, treatment duration), the immune status of treated patients, and finally the possible side-effects of the treatment.

Novel Chromone Alkaloids from *Schumanniphyton magnificum*

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Abstract: Six chromone alkaloids were isolated from the root bark of *Schumanniphyton magnificum* Harms. Three of these were identified as the known alkaloids schumanniphytine, schumannificine and *N*-methylschumannificine. The other three alkaloids are novel and were named isoschumanniphytine, anhydroschumannificine and *N*-methylanhydroschumannificine. In addition it was seen that the *N*-methylschumannificine isolated consisted of a mixture of two isomers and an alternative structure for schumanniphytine has been proposed.

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As already suggested concerning sergeolide, modifications of the molecule are necessary in order to attenuate its toxicity, while preserving its antimalarial properties (5).

However, a search for new compounds of the quassinoid family appears relevant, because of possible isolation of similar active molecules possessing high antimalarial activity versus reduced toxicity.

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Introduction

Schumanniphyton magnificum Harms. (Rubiaceae) is found in Nigeria and its uses there have been reported by Okogun et al. (1). There is current scientific interest in the plant because of the traditional use of it as a remedy for snake bites.

Little chemical work has been done on the genus. Schlittler and Spitaler (2) investigated *S. problematicum* and isolated the chromone noreugenin **1** and three alkaloids, schumanniphytine **2** and two unnamed piperidin-2-ones **3a**, **3b**. Okogun et al. (1) examined the root bark of *S. magnificum* and isolated noreugenin **1** and two alkaloids with a novel structure, schumannificine **4a** and *N*-methylschumannificine **4b**. They also mentioned the presence of other alkaloids which they had not yet characterised.