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In Vitro and In Vivo Activities of Atalaphillinine and Related Acridone Alkaloids against Rodent Malaria

HISASHI FUJIOKA,[1]* YUKIHIRO NISHIYAMA,[2] HIROSHI FURUKAWA,[3] AND NOBUO KUMADA[1]

Department of Medical Zoology[1] and Laboratory of Virology, Research Institute for Disease Mechanism and Control,[2] Nagoya University School of Medicine, Showa-ku, Nagoya 466, and Faculty of Pharmacy, Meijo University, Tempaku-ku, Nagoya 468,[3] Japan

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Thirty acridone alkaloids obtained from *Citrus*, *Glycosmis*, or *Severinia* plants (members of the family *Rutaceae*) were tested for their antimalarial activities in vitro and in vivo. At a concentration of 10 μg/ml in vitro, seven of these alkaloids suppressed 90% or more of *Plasmodium yoelii*, which causes malaria in rodents. Atalaphillinine, when injected intraperitoneally in a daily dose of 50 mg/kg for 3 days into mice infected with 10^7 erythrocytes parasitized with *Plasmodium berghei* or *Plasmodium vinckei*, completely suppressed the development of malaria parasites, with there being no obvious acute toxic effects from the tested dose.

In 1957 the appearance of strains of *Plasmodium falciparum* showing resistance to chloroquine was reported in South America (Colombia) and Asia (along the Cambodia [Kampuchea]-Thailand border area). The resistance was confirmed in East Africa by 1977 and has now been confirmed in more than 40 countries in South America, Asia, and Africa (7). Several hundred million people in the world still suffer from malaria, but the antimalarial drugs that are in use have limitations in their efficacy spectra (19). In particular, chloroquine resistance in *P. falciparum* has increasingly become an important problem around the world for the prophylaxis and treatment of malaria (9). Therefore, it is urgent that novel antimalarial agents be added to our chemotherapeutic armamentaria to cope with this dreadful disease.

Bai-Yu, which is extracted from the fruits of the plant *Citrus grandis*, is well-known as an important folk medicine in Taiwan, Republic of China (24). Acronycine, an acridone alkaloid that was first isolated from the Australian scrub ash *Acronychia baueri* (17); modified acronycine analogs; and acronycine derivatives have been studied for their antimicrobial activities; some derivatives of acronycine have been reported to be effective against *Trichomonas vaginalis* infection in mice (16). To our knowledge, however, no acridone alkaloids have been monitored for their antimalarial activities. In the present study, we examined the anti-*Plasmodium yoelii* activities of 30 acridone alkaloids, including new compounds (4, 20–24), and found that atalaphillinine exhibits antimalarial activity both in vitro and in vivo.

MATERIALS AND METHODS

Rodent malaria strains. *P. yoelii* subsp. *nigeriensis* was received from M. Suzuki of the Department of Parasitology, Gunma University School of Medicine. *Plasmodium berghei* NK65 was supplied by H. Ohtomo of the Department of Parasitology, Gifu University School of Medicine. *Plasmodium vinckei* subsp. *vinckei* was received from K. Tanabe of the Department of Medical Zoology, Osaka City University Medical School.

Maintenance of parasites. *P. yoelii* was maintained by passage through the blood of ICR mice and was transmitted frequently through the mosquito *Anopheles stephensi*. *P. berghei* and *P. vinckei* were maintained by passage through the blood of ICR mice.

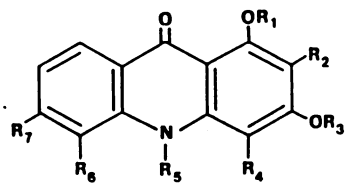
Sources of acridone alkaloids. Most of the compounds used in this study were isolated from the root barks of *Citrus*, *Glycosmis*, or *Severinia* plants (4, 20–24; compounds 1 to 30, Fig. 1). Three of these compounds were synthesized.

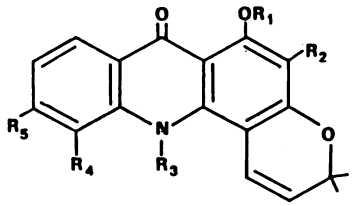
Infected erythrocytes. Male mice (ICR line; specific pathogen free; weight, 18 to 20 g) were used throughout the experiment. Infected erythrocytes (IE) were obtained by cardiac puncture, while the mice were under deep ether anaesthesia, from mice that were infected intraperitoneally (i.p.) with *P. yoelii*. The blood was suspended in an equal volume of RPMI 1640 medium (GIBCO Laboratories) with 20 IU of heparin per ml and centrifuged at 200 × *g* for 5 min. The erythrocytes were washed twice with the same medium.

Cultivation procedure. The cultivation procedure was based on the method of Jensen and Trager (5). For all cultures RPMI 1640 medium with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; 5.94 g/liter) and $NaHCO_3$ (42 ml of a 5% solution per liter) was used. It was supplemented with serum (100 ml/liter) from Wistar rats and neomycin (50,000 IU/liter) to give the complete medium.

Test procedure in vitro. The acridone alkaloids were first dissolved in dimethyl sulfoxide (DMSO) and diluted with RPMI 1640 medium before they were used in tests. Chloroquine diphosphate was dissolved in and diluted with RPMI 1640 medium before it was used in the tests. The concentration of DMSO in the test never exceeded 0.17%, which was demonstrated by the control experiment to have no harmful effect on infectivity. Diluted acridone alkaloids (10 μl each) were dispensed into plastic petri dishes (diameter, 35 mm), to give a final concentration of 10 μg/ml. To each dish was added 2 ml of mouse erythrocytes (8% erythrocyte suspension containing 8% IE and 92% uninfected, washed erythrocytes), and these were cultured for 24 h. All tests were performed in duplicate. After cultivation, erythrocytes were washed twice with RPMI 1640 medium by centrifugation at 200 × *g* for 5 min. The packed cells were diluted with the fresh medium to make an approximately 5% erythrocyte suspension. The mice were inoculated intravenously (i.v.) with 0.1 ml of this erythrocyte suspension via the tail or ophthalmic veins. The use of the i.v. route has been found to give less scatter in parasitemia levels (12). The parasites were counted every 24 h in Giemsa-stained thin films made

* Corresponding author.



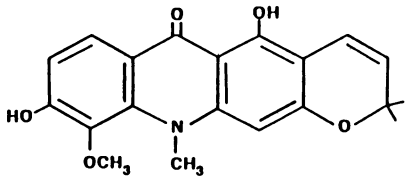


Compound No.	R_1	R_2	R_3	R_4	R_5	R_6	R_7
1	H	H	H	H	H	H	H
2	H	H	H	H	CH_3	OCH_3	OCH_3
3	H	H	CH_3	H	CH_3	OH	OCH_3
4	H	H	CH_3	H	CH_3	OCH_3	OH
5	H	H	CH_3	H	CH_3	OCH_3	OCH_3
6	H	H	CH_3	C_5H_9	CH_3	H	H
7	H	H	CH_3	C_5H_9	CH_3	OH	H
8	H	H	CH_3	C_5H_9	CH_3	OCH_3	OH
9	H	H	CH_3	OCH_3	CH_3	OH	H
10	H	C_5H_9	H	C_5H_9	CH_3	OH	H
11	H	OCH_3	CH_3	OCH_3	CH_3	OCH_3	OH
12	CH_3	H	CH_3	H	CH_3	H	H
13	CH_3	H	CH_3	H	CH_3	OCH_3	OCH_3
14	$COCH_3$	H	$COCH_3$	H	H	H	H
15	$COCH_3$	H	$COCH_3$	H	CH_3	H	H

Compound No.	R_1	R_2	R_3	R_4	R_5
16	H	H	H	H	H
17	H	H	H	OH	H
18	H	H	CH_3	H	H
19	H	H	CH_3	OH	H
20	H	H	CH_3	OCH_3	OH
21	H	H	CH_3	OCH_3	OCH_3
22	H	C_5H_9	H	H	H
23	H	C_5H_9	H	OH	H
24	H	C_5H_9	CH_3	H	H
25	H	C_5H_9	CH_3	OH	H
26	CH_3	H	CH_3	H	H
27	CH_3	H	CH_3	OCH_3	H
28	CH_3	H	CH_3	OCH_3	OCH_3

C_5H_9 = prenyl group

29



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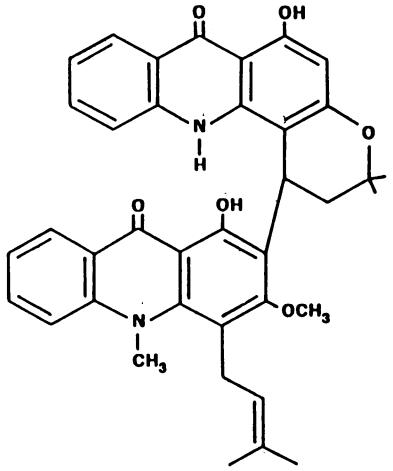

FIG. 1. Structures of the acridone alkaloids tested. 1, synthetic compound; 2, glandisine-II; 3, glandisine-I; 4, citpressine-I; 5, citpressine-II; 6, *O*-methylglycocitrine-II; 7, glycocitrine-I; 8, grandisinine; 9, citrusinine-I; 10, *N*-methylatalaphilline; 11, glyfoline; 12, 1,3-*O*-methyl-*N*-methylacridone; 13, 1,3,5,6-*O*-methyl-*N*-methylacridone; 14, synthetic compound; 15, synthetic compound; 16, *des*-*N*-methylnoracronycine; 17, atalaphillidine; 18, noracronycine; 19, 5-hydroxynoracronycine; 20, citracridone-I; 21, citracridone-II; 22, severifoline; 23, atalaphillinine; 24, *N*-methylseverifoline; 25, 5-hydroxy-*N*-methylseverifoline; 26, acronycine; 27, 5-methoxyacronycine; 28, dimethoxyacronycine; 29, honyumine; 30, glycobismine-A.

from blood obtained from the tails of the mice. A group of 10 male mice was used to test each compound.

Analysis of results. Standard curves were drawn as follows (3). A group of five male mice was inoculated i.v. with 10^3 to 10^7 IE, and every 24 h thin films were prepared from blood obtained from the tails of the mice. The development of parasitemia was measured after Giemsa staining (Fig. 2). The number of infective parasites in the mice, after they were treated with the various compounds for 24 h, was estimated from the standard curves. The percentage of inhibition was calculated from the following equation: percent inhibition = 100 − {[the estimated number of infective parasites treated with compounds/the estimated number of infective parasites treated with no compound (control)] × 100}.

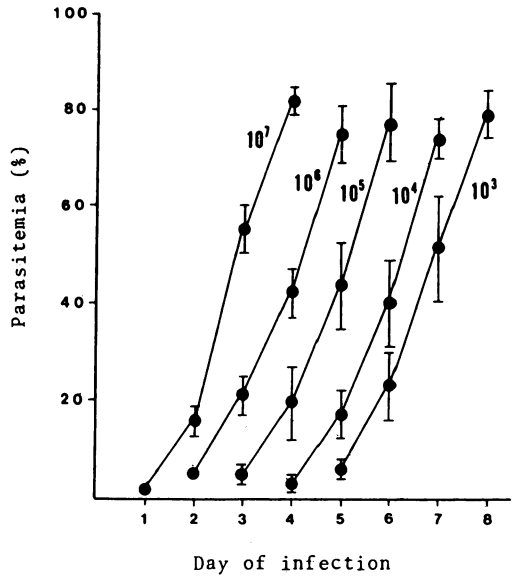

FIG. 2. Influence of a 10-fold decrease in inoculum size of i.v. infection on the course of parasitemia with *P. yoelii* in mice (values are percent parasitemia ± standard error; $n = 5$).

Test procedure in vivo. Atalaphillinine was also tested for its prophylactic activity against *P. berghei* and *P. vinckei*. This compound was dissolved in DMSO (50 mg/ml) and diluted 10-fold with olive oil before it was used in the tests. Mice were inoculated i.p. with approximately 10^7 IE and then received 50 mg of the compound per kg i.p. at 6, 24, and 48 h after the start of infection. Control mice were treated with DMSO-olive oil only. Daily counts were made of IE from the second day of treatment until the remission of disease, i.e., until the parasitemia was cleared. Five infected mice were used in each experiment.

RESULTS

Effects of acridone alkaloids on *P. yoelii* in vitro. Of the 30 acridone alkaliods tested, 7 suppressed 90% or more of intraerythrocytic *P. yoelii* at a concentration of 10 μg/ml (Table 1). They proved to be equally or more highly effective than chloroquine diphosphate in vitro (Table 1).

Prophylactic activity of atalaphillinine. Among the seven compounds with good suppressive activities, only atalaphillinine was tested for in vivo activity. It had marked prophylactic activity against *P. berghei* and *P. vinckei* infections in mice (Fig. 3). By days 4 to 5 after the infection, very few IE were seen in the blood smears, and by day 9 or 10 IE completely disappeared. On day 30 no sign of recrudescence was observed. In addition, no obvious acute toxic effect from the tested dose was observed in the mice for 30 days after administration. All the control mice died on days 6 to 8.

DISCUSSION

The malaria parasites in rodents, particularly *P. berghei*, have proved to be very useful for the detection of antimalarial compounds (10). It is generally considered that infection with *P. berghei* in laboratory mice is a valuable model for the primary screening of drugs against human malaria (8). In the search for new drugs against schizonts in blood, over 20,000 compounds have been screened by using *P. berghei*; and several compounds, such as $WR_{30,090}$, $WR_{142,490}$ (4-quinolinemethanols), and $WR_{122,455}$ (a 9-phenanthrenemethanol), have been shown to have antimalarial activities (1, 11, 15). Recently, quassinoids and sesquiterpenes obtained from some simaroubaceous and artemisia plants have also been found to have potent activities against human and rodent malaria (6, 13, 14). Results of the present study confirm that seven kinds of acridone alkaloids obtained from some rutaceous plants, i.e., glycocitrine-I, *des-N*-methylnoracronycine, atalaphillinine, 5-hydroxy-*N*-methylseverifoline, atalaphillidine, *N*-methylatalaphilline, and glycobismine-A, have antimalarial activities that are comparable to or greater than that of chloroquine diphosphate. 5-Hydroxynoracronycine and acronycine suppressed almost 90% of *P. yoelii* in vitro (Table 1). No obvious relationship between the structures of the acridone alkaloids tested and antimalarial activity was apparent. However, it was interesting to find out that the binary acridone alkaloid glycobismine-A also has a potent antimalarial activity.

From the results shown in Fig. 3, the development of malarial parasites was completely inhibited by the prophylactic administration of atalaphillinine. This compound was injected i.p. into mice in a daily dose of 50 mg/kg for 3 days with no obvious toxic effects, and no acute toxic effect was observed when it was injected i.p. into mice in a single dose of 150 mg/kg. The prophylactic activity and toxicity of atalaphillinine in mice should be investigated further.

Among the acridone alkaloids, only acronycine and its derivatives and analogs have been reported to have biological and biochemical effects against mammalian cells, proto-

TABLE 1. In vitro antimalarial activities of acridone alkaloids

Compound no. and name	% Inhibition with a dose of 10 μg/ml[a]
1. Synthetic compound	32 ± 11
2. Glandisine-II	78 ± 15
3. Glandisine-I	60 ± 17
4. Citpressine-I	23 ± 5
5. Citpressine-II	27 ± 6
6. *O*-Methylglycocitrine-II	26 ± 10
7. Glycocitrine-I	96 ± 4
8. Grandisinine	26 ± 10
9. Citrussinine-I	30 ± 4
10. *N*-Methylatalaphilline	91 ± 3
11. Glyfoline	0
12. 1,3-*O*-Methyl-*N*-methylacridone	80 ± 5
13. 1,3,5,6-*O*-Methyl-*N*-methylacridone	0
14. Synthetic compound	39 ± 18
15. Synthetic compound	0
16. *des-N*-Methylnoracronycine	95 ± 5
17. Atalaphillidine	90 ± 5
18. Noracronycine	12 ± 5
19. 5-Hydroxynoracronycine	88 ± 2
20. Citracridone-I	15 ± 3
21. Citracridone-II	0
22. Severifoline	0
23. Atalaphillinine	94 ± 4
24. N-Methylseverifoline	25 ± 3
25. 5-Hydroxy-*N*-methylseverifoline	92 ± 8
26. Acronycine	85 ± 5
27. 5-Methoxyacronycine	75 ± 8
28. Dimethoxyacronycine	31 ± 5
29. Honyumine	0
30. Glycobismine-A	96 ± 4
Chloroquine diphosphate	94 ± 4

[a] The mean ± standard error of the measurements in 10 mice calculated by standard curves (cf. text).

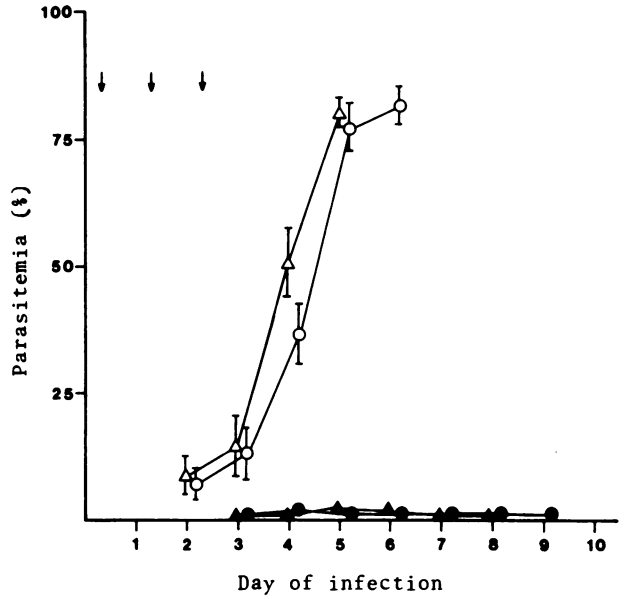

FIG. 3. Anti-*P. berghei* and anti-*P. vinckei* activities of atalaphillinine in mice. The curves refer to mice infected with *P. berghei* and treated with 50 mg of atalaphillinine per kg of body weight given 3 times (●); with *P. vinckei* and treated with 50 mg of atalaphillinine per kg of body weight given 3 times (▲); with *P. berghei* (control) (○); and with *P. vinckei* (control) (△). Arrows indicate injections of atalaphillinine at intervals of 6, 24, and 48 h after mice were infected i.p. with malarial parasites.

zoans, and helminths (2, 16–18). A number of acronycine derivatives have been found to have a curing effect on *Trichomonas vaginalis* infection in mice. But none of these compounds, including noracronycine and *des*-N-methylnoracronycine, exhibited any activity against *Entamoeba histolytica* or *Syphacia obvelata* in vivo (16). This is the first report in which the high activities of acridone alkaloids against rodent malaria have been described in vitro and in vivo. However, the antimalarial mechanisms of action of atalaphillinine and other acridone alkaloids remain to be elucidated. Further biochemical and pharmacological studies should be carried out.

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