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Licochalcone A, a New Antimalarial Agent, Inhibits In Vitro Growth of the Human Malaria Parasite *Plasmodium falciparum* and Protects Mice from *P. yoelii* Infection

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Licochalcone A, isolated from Chinese licorice roots, inhibited the in vitro growth of both chloroquine-susceptible (3D7) and chloroquine-resistant (Dd2) *Plasmodium falciparum* strains in a [^{3}H]hypoxanthine uptake assay. The growth inhibition of the chloroquine-resistant strain by licochalcone A was similar to that of the chloroquine-susceptible strain. To examine the activity of licochalcone A on the different asexual blood stages of the parasite, licochalcone A was added to highly synchronized cultures containing rings, trophozoites, and schizonts. The growth of the parasites at all stages was inhibited by licochalcone A. The in vivo activity of licochalcone A was tested in a mouse model of infection with *P. yoelii*. Licochalcone A administered either intraperitoneally or orally for 3 to 6 days protected the mice from the otherwise lethal *P. yoelii* infection. These results demonstrate that licochalcone A exhibits potent antimalarial activity and might be developed into a new antimalarial drug.

Malaria is one of the most serious health problems in many parts of the world, particularly in Africa, Asia, and Latin America. In 1990 the World Health Organization (33) estimated the global incidence of malaria to be on the order of 110 million clinical cases annually, with some 270 million people infected and 1 million to 2 million people dying from the disease each year. More than 80% of the world's malaria cases are found in Africa, where approximately 1 million children die from the disease every year (28). The situation is further complicated by the spread of drug-resistant parasites in many parts of the world where *Plasmodium falciparum* is endemic (12, 16, 18, 29). Today, in some areas of the world such as Thailand, multiple drug resistance is so prevalent that there is little to choose from for the prophylaxis or treatment of malaria (28). Another serious problem encountered in malaria chemotherapy is the adverse effects of amodiaquine and the sulfonamide-pyrimethamine combinations, which have been used as effective first-line drugs (15). The recent discovery of chloroquine resistance in some *Plasmodium vivax* parasites adds to the problem (26). Therefore, there is a great need for the development of effective and safe drugs for the prophylaxis and treatment of malaria.

A number of the available antimalarial drugs have originated from plant sources, and the potential of plants for the production of new antiprotozoal agents has recently been emphasized (14, 19–21). The discovery of the antimalarial property of artemisinin and its analogs is such an example (9, 23).

Licorice roots under the name of Gan Cao have been used in the People's Republic of China for the treatment of gastric and duodenal ulcer, bronchial asthma, Addison's disease, food and drug poisoning, and skin diseases such as eczema and urticaria (30). We recently reported that an ethanolic extract of the Chinese licorice roots obtained from the Xinjiang Province of the People's Republic of China inhibited the in vitro growth of *Leishmania major* and *Leishmania donovani* promastigotes. A bioassay-guided fractionation revealed that licochalcone A was the major active principle (2). Similarly, our preliminary experiments demonstrated that the extract of Chinese licorice roots inhibited the in vitro growth of the human malaria parasite *P. falciparum*. Here we report that licochalcone A inhibits the in vitro growth of the human malaria parasite *P. falciparum* and controls *Plasmodium yoelii* infection in mice.

MATERIALS AND METHODS

Licochalcone A. Licochalcone A was isolated from Chinese licorice roots as described previously (2). The chemical structure of licochalcone A is shown in Fig. 1. Licochalcone A was also synthesized in our laboratory by previously published methods (8, 25). Furthermore, larger amounts of licochalcone A were synthesized by the same method according to good manufacturing practices rules by Clauson-Kaas, Farum, Denmark. The identity of the synthetic licochalcone A was established by recording the ^{1}H nuclear magnetic resonance (200 MHz) and the ^{13}C nuclear magnetic resonance (50 MHz) spectra. The purity of licochalcone A was ensured by combustion analysis and high-pressure liquid chromatography (HPLC). The purity determined by HPLC was greater than 99.4%. Licochalcone A either was dissolved in 20 μl of 99% (vol/vol) ethanol and added to 980 μl of medium 199 or was suspended in an aqueous 1% carboxymethyl cellulose (CMC) solution. Licochalcone A purified from licorice roots was used for the in vitro studies, while synthetic licochalcone A was used for the in vivo studies.

In vitro antimalaria studies. (i) Parasite cultures. A chlo-

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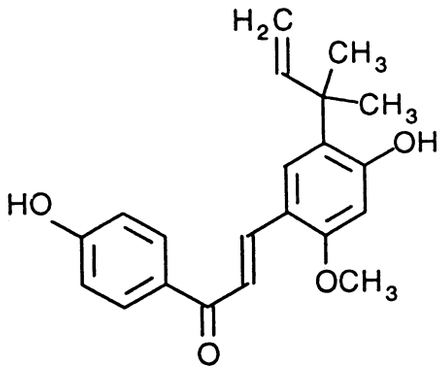

FIG. 1. Chemical structure of licochalcone A.

roquine-susceptible strain (3D7) and a chloroquine-resistant strain (Dd2) of *P. falciparum* were used. Both strains were kindly provided by D. Walliker (Edinburgh, Scotland). *P. falciparum* was kept in continuous culture by a modification of the method originally described by Trager and Jensen (32). Human peripheral blood was drawn into 10-ml Vacutainers containing citrate-phosphate-glucose, and the Vacutainers were stored at 4°C for 2 to 4 weeks before use. At the day of use, the cells were washed twice in RPMI 1640 medium containing 5% type A-positive serum, HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; 5.94 g/liter), and sodium bicarbonate (7.5%; 31 ml/liter). After each wash the supernatant and the buffy coat containing leukocytes were removed. The parasites were cultured in Nunc culture flasks (Nunc, Roskilde, Denmark) containing 200 μl of packed erythrocytes in 5 ml of the medium described above. The medium was changed every 24 h, and packed erythrocytes were supplied twice weekly. The parasite cultures were carried out at 37°C in an atmosphere of 2% oxygen–5% carbon dioxide–93% nitrogen.

(ii) Growth inhibition assay. The effects of licorice root extract and licochalcone A on *P. falciparum* were assessed by a modification of the method originally described by Jensen et al. (7). Fifty microliters of parasitized erythrocytes (parasitemia of approximately 1%) at a concentration of 5×10^8 cells per ml and 50 μl of the medium containing licochalcone A (0.1 to 10 μg/ml) were added to each well of 96-well flat-bottom microtiter plates (Nunc). The cultures were then incubated for 48 h. Twenty-four hours before termination of the cultures, 20 μl of [^{3}H]hypoxanthine (40 μCi/ml; New England Nuclear, Boston, Mass.) was added to each well. The cultures were harvested on filter paper by a cell harvester (Skatron, Lierbyen, Norway), and the cells were counted in a scintillation counter (Minaxi Tri-Carb 4000; United Technologies, Packard Instrument Co. Inc., Rockville, Md.). All cultures were performed in triplicate. Control cultures with uninfected erythrocytes and infected erythrocytes in medium without the licorice extract or licochalcone A were always performed in parallel with the test cultures. The percent growth inhibition was determined as follows: percent growth inhibition = {1 − [cpm (or level of parasitemia) in treated *P. falciparum* parasites/cpm (or level of parasitemia) in untreated *P. falciparum* parasites]} × 100.

In some experiments, thin smears of parasite cultures were stained with Giemsa and the level of parasitemia was determined by microscopic counting of 2,500 cells.

(iii) Stage specificity assay. A chloroquine-resistant strain (Dd2) of *P. falciparum* was used for testing the stage specificity of licochalcone A. Parasites were synchronized to the young ring stage by sorbitol lysis by a modification of previously described methods (4, 11). Briefly, parasitized erythrocytes were centrifuged at 2,500 rpm for 5 min, the supernatant was discarded, and the pellet was resuspended in 5 ml of aqueous 5% D-sorbitol for 5 min at room temperature. After an additional centrifugation, 5 ml of the RPMI 1640 medium was added to the pellet. By this procedure we obtained about 90% parasites at the young ring stage. Cultures were reestablished in 96-well flat-bottom microtiter plates (Nunc) by the addition of uninfected erythrocytes and medium to give a 12.5% hematocrit with an appropriate starting level of parasitemia (0.1 to 0.5%). After synchronization, one of the cultures was immediately treated with licochalcone A and was incubated for 20 h (young ring to trophozoite stages) with [^{3}H]hypoxanthine. At 20 h after synchronization, another culture was treated with different concentrations of licochalcone A and was incubated for 24 h (trophozoite to schizont stages) with [^{3}H]hypoxanthine. At 44 h after synchronization, the last culture was treated with different concentrations of licochalcone A and was incubated for 12 h (schizont to young ring stages) with [^{3}H]hypoxanthine.

Thin smears of parasite cultures were stained with Giemsa, and the level of parasitemia and the number of rings, trophozoites, and schizonts were determined by counting the number of parasites per 5,000 cells under microscope. The percent growth inhibition was determined as described above.

For comparison, the effects of chloroquine were tested in parallel experiments.

In vivo antimalaria studies. (i) Parasite and animal. *P. yoelii* YM, kindly provided by D. Walliker, was used in the study. This strain is lethal to infected mice within 6 to 9 days. BALB/c female mice (age, 8 weeks; body weight, 20 g) were used throughout the study.

(ii) Test procedures. The study was carried out by using three different experimental setups. The first was a modification of the "4-Day Suppressive Test" originally described by Peters (17). Briefly, animals were inoculated intraperitoneally with 10^6 parasitized erythrocytes in 0.2 ml. These parasitized erythrocytes were obtained from the blood of highly infected mice (average, 30% rising parasitemia); blood was diluted in 0.9% NaCl to give 5×10^6 parasites per ml. For each experiment, mice were randomly assigned to a given treatment group (five mice in each group). The day of infection is termed D0, and succeeding days of infection are termed D+1, D+2, etc. Licochalcone A was first dissolved in dimethyl sulfoxide and was then diluted in polyoxyethylene 23-lauryl ether (Brij 35)–normal saline. Licochalcone A (0.2 ml) was injected intraperitoneally into each animal at dosages of 5, 10, and 15 mg/kg of body weight given twice per day. The first administration started 3 h after infection on D0, once on D0, and twice daily from D+1 to D+3, while control mice received 0.2 ml of normal saline. As a control, chloroquine was given intraperitoneally in 0.2 ml at dosages of 0.5, 2.5, and 5 mg/kg of body weight once per day. From D+4 of infection, thin blood smears were made from the tail blood of mice every second day. The levels of parasitemia in mice, as seen in Giemsa-stained smears, were assessed. In the second series of experiments, licochalcone A at a dose of 10 mg/kg was given to mice 4 times per day from D+4 to D+7 of infection. In the third series of experiments, the effect of licochalcone A was tested by administration of the compound through the oral route. Licochalcone A was suspended in 1% CMC solution and was given to mice once per day from 3 h after infection up to D+5 of infection.

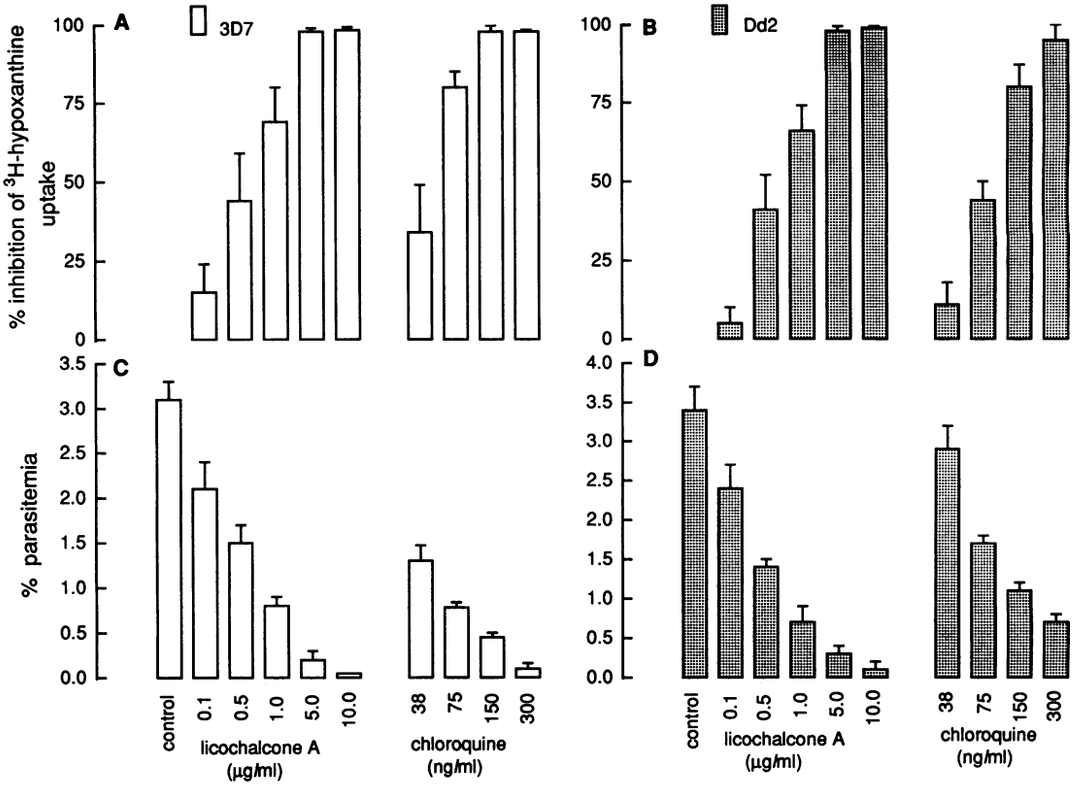

FIG. 2. Effects of licochalcone A and chloroquine on the in vitro growth of chloroquine-susceptible (3D7) (A) and chloroquine-resistant (Dd2), (B) *P. falciparum*. Data are from six experiments and are given as percent inhibition of [^{3}H]hypoxanthine uptake (mean ± 95% confidence intervals). The mean levels of incorporation of [^{3}H]hypoxanthine in control cultures were 15.8 kcpm (3D7) and 26.5 kcpm (Dd2). The effects of licochalcone A and chloroquine on the level of parasitemia of 3D7 (C) and Dd2 (D) on in vitro culture are also shown. Data are given as the mean level of parasitemia ± 95% confidence intervals from five experiments.

Statistical analysis. Paired two-tailed *t* test was used for analysis of the data. *P* values of <0.05 were considered significant.

RESULTS

In vitro studies. Figures 2A and B show that licochalcone A exhibited a strong inhibitory effect on the [^{3}H]hypoxanthine uptake of both the chloroquine-susceptible (3D7) strain and the chloroquine-resistant (Dd2) strain. The *P* values for the effect of licochalcone A at concentrations of 0.1 μg/ml (3D7) or 0.5 μg/ml (Dd2) and greater in compared with the effect of the control were less than 0.05. Figures 2C and D show a dose-dependent reduction in the level of parasitemia by licochalcone A in both strains 3D7 and Dd2. The *P* values for the effect of licochalcone A at concentrations of 0.1 μg/ml and greater compared with the effect of the control treatment were less than 0.05. The effect of licochalcone A on the in vitro growth of *P. falciparum* was similar for both strains, while the inhibitory effect of chloroquine on the 3D7 strain was much stronger than the effect on the Dd2 strain.

The stage specificity of licochalcone A was studied by exposing highly synchronized parasite cultures to the compound during different stages of the parasite's life cycle. The effect of the compound was examined both by microscopic counting and by measuring the level of [^{3}H]hypoxanthine incorporation. The parasites incubated in medium passed from the ring stage to the trophozoite stage in the time period between 0 and 20 h after synchronization. In the time period from 20 to 44 h after synchronization, approximately half of the parasites developed into schizonts, and from 44 to 56 h after synchronization, parasites matured from trophozoites to schizonts and some parasites invaded new erythrocytes and were detected as rings. Figures 3A and B show that the treatment with licochalcone A and chloroquine inhibited [^{3}H]hypoxanthine incorporation of the parasites and that parasites at all stages were affected by licochalcone A. The *P* values for the effect of licochalcone A at concentrations of 0.1 μg/ml and greater compared with the effect of the control treatment were less than 0.05. This conclusion is further illustrated by the results presented in Fig. 3C and D, which show similar levels of inhibition of parasitemia during the three different time periods.

In vivo studies. Table 1 shows that licochalcone A injected intraperitoneally at 5, 10, and 15 mg/kg twice per day for 3 days markedly reduced the level of parasitemia in mice infected with *P. yoelii* YM. All of the control animals died on D+8 of infection. Most of the animals in the groups receiving licochalcone A at 5 and 10 mg/kg survived for up to 3 weeks after the initiation of infection. Four of five mice from the group receiving 15 mg of licochalcone A per kg survived the infection and cleared the parasites. In a parallel experiment, chloroquine-treated groups also had reduced levels of parasitemia. On D+21, one of five mice in the group receiving chloroquine at 0.5 mg/kg was alive, three of five mice in the group receiving chloroquine at 2.5 mg/kg were alive, and four of five mice in

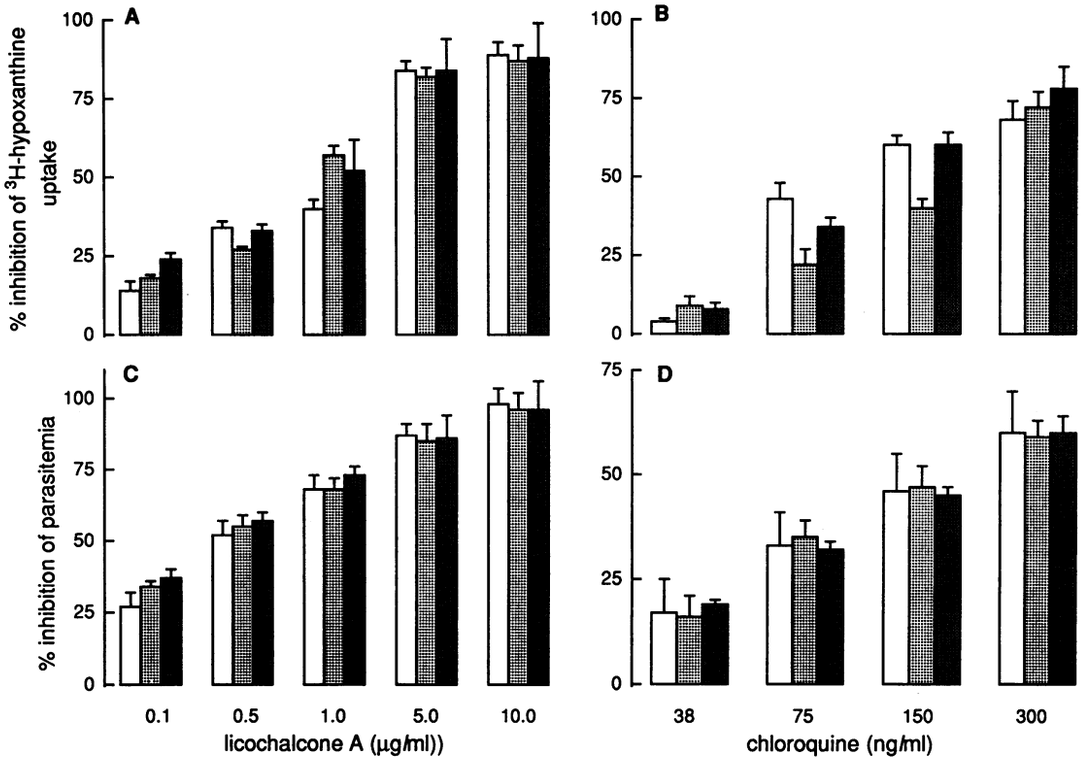

FIG. 3. Study on the stage-specific actions of licochalcone A (A) and chloroquine (B) on strain Dd2 determined by the level of [^{3}H]hypoxanthine incorporation. Data are from six experiments and are given as percent inhibition of [^{3}H]hypoxanthine uptake (mean ± 95% confidence intervals). The mean levels of incorporation in control cultures were 4.4 kcpm (0 to 20 h [□]), 6.3 kcpm (20 to 44 h [▨], and 4.9 kcpm (44 to 56 h [▩]). The stage-specific actions of licochalcone A (C) and chloroquine (D) on Dd2 strain were determined by microscopic counting of the level of parasitemia in the culture. Data are from six experiments and are given as percent inhibition of parasitemia (mean ± 95% confidence intervals).

the group receiving chloroquine at 5 mg/kg were alive. Figure 4 shows that 10 mg of licochalcone A per kg injected intraperitoneally four times daily from D+4 to D+7 of infection reduced the level of parasitemia from 14.2 to 6.6% on D+6 of infection. The level of parasitemia increased after the termination of the treatment. However, two of five mice survived after D+21 of infection, while all the control animals died on D+8 of infection.

Licochalcone A given orally at 450, 150, and 50 mg/kg once daily for 6 days starting 3 h after infection almost completely cleared the parasites from the mice, and all of the licochalcone A-treated mice were still alive on D+21 of infection, when the experiment was terminated (Table 2). All of the control mice died between D+7 and D+9 of infection.

DISCUSSION

Licochalcone A is one of the many flavonoids present in the Chinese licorice root, which, under the name of Gan Cao, is used in traditional Chinese medicine (24). However, the amount of licochalcone A present in the three *Glycyrrhiza* species accepted in the Chinese pharmacopoeia as sources for Gan Cao (*G. glabra*, *G. uralensis*, and *G. inflata*) has been demonstrated to vary considerably (24, 34). The structure of licochalcone A was first reported in 1975, but no biological activity was described (27). Later studies have revealed that licochalcone A exhibits antimicrobial (6) and antioxidant (13) activities. In addition, licochalcone A has been reported to

TABLE 1. Effects of intraperitoneal administration of licochalcone A and chloroquine on mice infected with *P. yoelii* YM[a]

Treatment regimen and drug doses (mg/kg)	% Reduction in parasite density[b]		Mortality on day 21 (no. of dead mice/ total no. of mice treated)	
	Expt 1	Expt 2	Expt 1	Expt 2
Controls[c]	0	0	5/5[d]	5/5[d]
Chloroquine				
0.5	94.3	ND[e]	4/5	ND
2.5	99.0	ND	2/5	ND
5	99.9	ND	1/5	ND
Licochalcone A				
5	95.0	92.4	5/5	5/5
10	96.6	95.0	5/5	4/5
15	93.2	99.8	1/5	1/5

[a] Treatment started 3 h after the infection (with half of the daily doses) and lasted for 3 days. Chloroquine was given once daily. Licochalcone A was given twice daily.

[b] The mean percent reduction in the level of parasitemia at day 4 was calculated as follows: [1 − (mean level of parasitemia in treated mice/mean level of parasitemia in control mice)] × 100.

[c] The mean level of parasitemia was 32.2% ± 2.2% in drug-free parasitized controls at day 4.

[d] All mice died on D+8 of infection.

[e] ND, not determined.

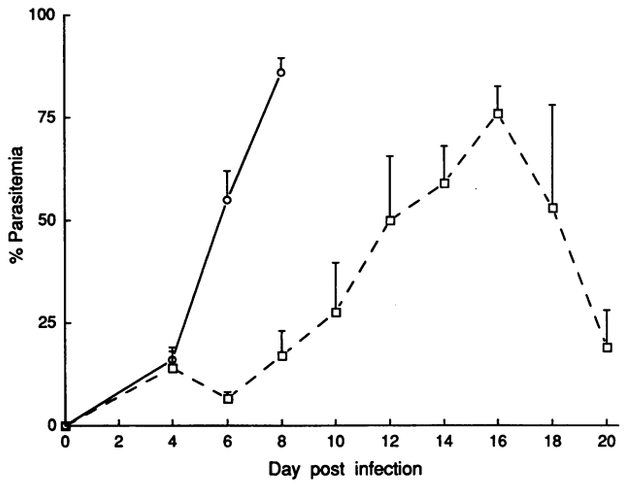

FIG. 4. Effect of licochalcone A on the level of parasitemia in mice parasitized by *P. yoelii* YM. Licochalcone A was given intraperitoneally at 10 mg/kg (□) four times daily over 4 days starting at D+4 after infection. The control mice received 0.2 ml of normal saline (○) intraperitoneally four times daily over 4 days. Each point represents the mean level of parasitemia ± standard error of the mean for five mice. All of the control mice died on D+9 of infection. One of five licochalcone A-treated mice died on D+16, another mouse died on D+18, and one more mouse died on D+20 of infection.

possess anti-inflammatory activity (31). Our previous studies showed that the extract of Chinese licorice roots and licochalcone A isolated from Chinese licorice roots have antileishmanial activities in vitro (2) as well as in vivo (3). The results from the present study clearly demonstrate that licochalcone A exhibits antimalarial activity against the human parasite *P. falciparum* in vitro and against *P. yoelii* infection in mice.

Using a [^{3}H]hypoxanthine uptake assay, we observed that the in vitro growth of both a chloroquine-susceptible (3D7) and a chloroquine-resistant (Dd2) *P. falciparum* strain was significantly inhibited by licochalcone A. The growth inhibition of the chloroquine-resistant strain by licochalcone A was similar to that of the chloroquine-susceptible strain. The 50% inhibition of both strains of *P. falciparum* by licochalcone A was at a concentration of about 0.6 μg/ml. The 50% inhibition of the chloroquine-susceptible strain by chloroquine was observed at a concentration of about 50 ng/ml, whereas that of the chloroquine-resistant strain was at a concentration of about 86 ng/ml. The results from parasitemia studies were similar to those from [^{3}H]hypoxanthine uptake tests.

To test whether licochalcone A had a differential effect on the morphologically distinguishable stages of the intraerythrocytic cycle of *P. falciparum*, we added the compound to highly synchronized parasite cultures. The activity of the compound was assayed by measuring its effect on the uptake of [^{3}H]hypoxanthine and by counting the number of infected erythrocytes microscopically. The results showed that the compound inhibited the parasites at all of the blood stages to the same degree, indicating that it acts on metabolic events important for the development of the parasite during the entire blood stage cycle.

The in vivo activity of licochalcone A against malaria parasites was tested by determining its ability to protect mice from the lethal effect of *P. yoelii* infection. Licochalcone A injected intraperitoneally at 5, 10, and 15 mg/kg twice per day for 3 days markedly reduced the level of parasitemia in mice infected with *P. yoelii* YM. Experiments were performed to examine whether licochalcone A could influence the course of a well-established infection in mice. In that study the mice were infected with *P. yoelii*, and at 4 days after infection the animals were injected intraperitoneally with 10 mg of licochalcone A per kg four times daily for 3 days. The results from that experiment showed that licochalcone A is able to reduce the level of parasitemia in the treated animals. However, the parasites were not completely cleared. Presumably, a longer period of treatment will be required to completely clear the parasites in animals with well-established infections.

The data presented in Table 2 demonstrate that oral administration of a suspension of licochalcone A efficiently controls an infection of *P. yoelii* in mice. The dosages used for the oral administration in the present study were higher than those used in the experiment in which licochalcone A was given by the intraperitoneal route. As shown in Table 2, the lowest concentration used (50 mg/kg) was as effective as the highest concentration used. Experiments with lower doses of the compound are in progress. The experiment also demonstrated that licochalcone A is absorbed through the gastrointestinal tract, an important advantage for the development of a new antimalarial drug.

Preliminary experiments on the potential toxicity of licochalcone A in vivo have shown that licochalcone A at concentrations of up to 1,000 mg/kg in 1% CMC administered orally to rats once daily for 2 weeks did not cause any observable signs of toxicity in the animals (unpublished data).

In the search for new antimalarial agents, a number of laboratories throughout the world are investigating plants used

TABLE 2. Effect of oral administration of licochalcone A on mice infected with *P. yoelii* YM[a]

Treatment regimen and dose	% Parasitemia (mean ± SEM) on:			Mortality on D+21 (no. of dead mice/ total no. of mice treated)
	D+4	D+6	D+8	
Control 1 (no oral administration)	42 ± 2.3	77 ± 1.7	ND[b]	5/5
Control 2 (1% CMC oral administration)	39 ± 4.0	75 ± 2.6	ND[b]	5/5
Licochalcone A				
50 mg/kg/day	0.06 ± 0.02	0.04 ± 0.01	0.02 ± 0.01	0/5
150 mg/kg/day	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0/5
450 mg/kg/day	0.024 ± 0.01	0.02 ± 0.01	0.01 ± 0.002	0/5

[a] Treatment started 3 h after the infection, was given once daily, and lasted for 6 days until D+5 of infection. Each group contained five mice.
[b] ND, not determined because the mice died.

in traditional medicine for their active constituents. In recent years, some antimalarial agents such as bruceantin (5, 16), digitolutin (10), febrifugine (22), and artemisinin (9, 23) have been identified. Artemisinin (Qinghaosu) was isolated from *Artemisia annua*, which is used in traditional Chinese medicine for the treatment of malaria. Artemisinin and its derivatives such as dihydroqinghaosu, artemether, sodium artesunate, and arteether, have proved to be effective in the treatment of cerebral malaria caused by chloroquine-resistant strains of *P. falciparum* (1, 9, 22, 23). It thus can be concluded that active principles isolated from plants used in traditional medicine offer new leads for the development of drugs that can be used to eradicate malaria.

In conclusion, the data presented in this report demonstrate that licochalcone A exhibits strong antimalarial activity. Licochalcone A inhibited the in vitro growth of both chloroquine-susceptible and chloroquine-resistant *P. falciparum* strains and protected mice from *P. yoelii* infection. The results presented in this report together with our previous findings on the antileishmanial activity of licochalcone A (2, 3) indicate that this chalcone could be the basis for the development of a new generation of antiparasitic drugs which can be used for the treatment of a number of diseases caused by protozoan parasites. A number of analogs of licochalcone A have been synthesized in our laboratories. Experiments are in progress to examine the effects of various analogs of licochalcone A and related oxygenated chalcones on malaria parasites both in vitro and in vivo and to elucidate the structure-activity relationships of these compounds.

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