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Antileishmanial Activity of Licochalcone A in Mice Infected with *Leishmania major* and in Hamsters Infected with *Leishmania donovani*

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This study was designed to examine the antileishmanial activity of the oxygenated chalcone licochalcone A in mice and hamsters infected with *Leishmania* parasites. Intraperitoneal administration of licochalcone A at doses of 2.5 and 5 mg/kg of body weight per day completely prevented lesion development in BALB/c mice infected with *Leishmania major*. Treatment of hamsters infected with *L. donovani* with intraperitoneal administration of licochalcone A at a dose of 20 mg/kg of body weight per day for 6 consecutive days resulted in a >96% reduction of parasite load in the liver and the spleen compared with values for untreated control animals. The [^{3}H]thymidine uptake by the parasites isolated from the treated hamsters was only about 1% of that observed in parasites isolated from the controls. Oral administration of licochalcone A at concentrations of 5 to 150 mg/kg of body weight per day for 6 consecutive days resulted in >65 and 85% reductions of *L. donovani* parasite loads in the liver and the spleen, respectively, compared with those of untreated control hamsters. These data clearly demonstrate that licochalcone A is a promising lead for the development of a new drug against leishmaniases.

The World Health Organization has identified leishmaniases as a major and increasing public health problem (23, 26). The visceral form of the disease, known as kala-azar, is widely distributed in many parts of the world, particularly Africa, Asia, Latin America, the Middle East, and the Mediterranean Basin (13, 23, 26). More than 350 million people in the world are at risk of infection with *Leishmania* parasites. Over 12 million people are infected with different species of the parasite, with over 400,000 new cases each year (1). Although a number of antileishmanial drugs are available (3, 9, 10), these drugs are in general toxic and expensive and require long-term treatment (23). A recent report on large-scale clinical resistance of kala-azar to antimonial agents, the most commonly used antileishmanial drugs (24), emphasizes the desperate need for new, effective, and safe chemotherapeutic agents for the treatment of leishmaniasis (18, 26). In recent years, the increase in the number of *Leishmania* infections in immunocompromised individuals such as those with AIDS and malignancies has added a new dimension to the problem (2, 6, 7).

Licochalcone A is one of the many flavonoids isolated from the roots of Chinese licorice. Licorice roots have been used in traditional Chinese medicine as a drug under the name Gan Cao (21). Our previous studies have proven that licochalcone A and some related chalcones inhibits the in vitro growth of both *Leishmania major* and *L. donovani* promastigotes and exhibits a remarkably strong ability to kill the intracellular parasites of both *L. major* and *L. donovani* amastigotes (5).

The present study was undertaken to investigate the potential of licochalcone A for the control of *Leishmania* infections in vivo. The results show that licochalcone A prevented lesion development in mice infected with *L. major* and inhibited the growth of *L. donovani* parasites in hamsters.

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MATERIALS AND METHODS

Animals. BALB/c female mice, 8 weeks old with a body weight of approximately 20 g, and male golden hamsters (*Mesocricetus auratus*), with a body weight of approximately 50 to 60 g, were used in this study.

Parasites. A World Health Organization reference vaccine strain of *L. major* originally isolated from a patient in Iran (MHOM/IL/67/LRC-L137), kindly provided by R. Behin, WHO Immunology Research and Training Centre, Lausanne, Switzerland, and a Kenyan strain of *L. donovani* (MHOM/KE/85/NLB 439), kindly provided by Kenya Medical Research Institute, Nairobi, Kenya, were used in these studies. The maintenance, cultivation, and isolation of promastigote-stage parasites have been described in detail elsewhere (5).

Licochalcone A. Two preparations of licochalcone A were used in these studies. The preparation used for the *L. major* experiments with mice and the intraperitoneally administered studies with hamsters infected with *L. donovani* was isolated from Chinese licorice roots in our laboratory as previously described (5). For the orally administered experiment with hamsters, synthetic licochalcone A was used. In order to ensure a steady supply of licochalcone A, we have synthesized the compound according to a previously published method (20). Furthermore, larger amounts of licochalcone A were synthesized by the same method according to Good Manufacturing Practices rules by Clauson-Kaas, Farum, Denmark. The synthesized licochalcone A possessed a melting point of 176 to 177°C. The identity of the synthetic licochalcone A was established by recording the ^{1}H nuclear magnetic resonance (200-MHz) and ^{13}C nuclear magnetic resonance (50-MHz) spectra. The purity of licochalcone A was ensured by satisfactory combustion analysis and high-pressure liquid chromatography (HPLC). The purity determined by HPLC was greater than 99.4%. Licochalcone A was either dissolved in 20 μl of 99% (vol/vol) ethanol and added to 980 μl of RPMI medium 199 or suspended in 1% carboxymethyl cellulose (CMC) solution.

Experimental procedures. (i) *L. major* infection in mice. On day 0, the mice received subcutaneous injections (in 0.05 ml of phosphate-buffered saline [PBS]) in the left hind footpad of 10^7 stationary-phase *L. major* promastigotes. The lesions developed in the footpad were measured with a dialcaliper and expressed as footpad thickness. The footpad thicknesses of mice were measured before infection, 7 days after infection (day 0), and every 3 days thereafter. Seven days after infection, the mice received licochalcone A or PBS intraperitoneally or intralesionally once a day. After 39 days of licochalcone A injections, some of the mice were killed and the footpads, the spleens, and the livers were removed. The parasite load ([^{3}H]thymidine uptake method) in the footpad was estimated by a modification of a previously described method (12). Briefly, the tissues were cut into small pieces and homogenized. The supernatants containing the released parasites were cultured in 15 ml of RPMI 199 containing 0.02 mg of gentamicin per ml, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 4 mM L-glutamine, and 20% heat-inactivated (56°C, 30 min) fetal calf serum in 25-cm^2, 50-ml culture flasks (Nunc, Roskilde, Denmark) at 28°C. After 3 days of incubation, 1 ml of the culture supernatant was centrifuged at 1,000 rpm for 10 min, washed with medium three times, and resuspended in 1 ml of fresh medium, and then 200 μl of the culture solution was transferred to round-bottom microtiter plates. The cultures were then pulsed with 1 μCi of [^{3}H]thymidine (New England Nuclear, Boston, Mass.). After 18 h of incubation, the cultures were harvested on filter paper with a cell harvester. The uptake of [^{3}H]thymidine by growing parasites was measured by liquid scintillation spectrometry. Impression smears of the footpads, the spleens, and the livers were also prepared. The slides were fixed with absolute methanol, stained with Giemsa stain, and examined by light microscopy. The parasite burdens in the footpads, the spleens, and the livers were determined (see Table 1).

(ii) *L. donovani* infection in hamsters. In order to determine the in vivo activity of licochalcone A in hamsters infected with *L. donovani*, the compound was administered either intraperitoneally or orally to the animals. A modification of an 8-day method for screening compounds against *L. donovani* in hamsters as described by Stauber et al. (22) was used in these studies. On day 0, 11 hamsters received intracardial injections of 10^9 stationary-phase *L. donovani* promastigotes in 0.1 ml of PBS. One hour after inoculation, one of the hamsters was killed. The liver and the spleen from this hamster were removed and weighed, and impression smears were made. The slides were fixed with absolute methanol, stained with Giemsa stain, and examined by light microscopy. Parasite loads in the liver and the spleen were determined. From day 1, five hamsters received 10 mg of licochalcone A per kg of body weight in 0.1 ml of PBS intraperitoneally twice a day for 6 days. Another five hamsters received the same volume of PBS as a control. In another experiment, groups of five hamsters each were orally administered 5, 50, and 150 mg of licochalcone A per kg of body weight per day or 0.5 ml of synthetic licochalcone A suspended in 1% CMC once a day for 6 days. The animals in the control group received 1% CMC in PBS orally. On day 8, all hamsters were sacrificed. Their livers and spleens were removed and weighed, and impression smears from the livers and spleens were prepared. The slides were fixed with absolute methanol, stained with Giemsa stain, and examined by light microscopy. The parasite load in the spleen was also estimated by the same method as described for the *L. major* study, using [^{3}H]thymidine uptake.

Statistical analysis. A paired two-tailed *t* test was used for the analysis of the data.

RESULTS

The antileishmanial activity of the synthetic licochalcone A was first tested in vitro on *L. major* and *L. donovani* promastigotes. The synthetic licochalcone A exhibited growth-inhibitory activity similar to that of the natural compound purified from the roots of licorice (data not shown).

Figure 1A shows that intraperitoneal administration of licochalcone A at doses of 2.5 and 5 mg/kg of body weight per day completely prevented lesion development in the mice infected with *L. major* compared with that in the control animals ($P < 0.05$). Figure 1B shows that intralesional administration of licochalcone A at doses of 1 and 2.5 mg/kg of body weight per day resulted in about 50% inhibition of lesion size in the mice infected with *L. major* compared with that in untreated animals ($P < 0.05$).

As shown in Fig. 2, the parasite loads as measured by [^{3}H]thymidine uptake in the footpads of mice infected with *L. major* were reduced by 80 and 75% when mice received licochalcone A intraperitoneally at doses of 2.5 and 5 mg/kg of body weight per day, respectively. Likewise, the intralesional administration of licochalcone A at a dose of 1 mg/kg of body weight per day resulted in about 50% inhibition of the parasite load in the footpads of mice infected with *L. major*.

A large number of parasites were observed in the impression smears of the footpads, spleens, and livers of the control mice. A considerably smaller number of parasites were observed in the footpads and the spleens of the mice treated with intralesional injections of licochalcone A at doses of 1 and 2.5 mg/kg of body weight per day. Only a very small number of parasites were found in the footpads of mice treated intraperitoneally with licochalcone A at doses of 2.5 and 5 mg/kg of body weight per day. No parasites were detected in the spleens and the livers from these animals (Table 1).

In the hamster model, the total number of *L. donovani* parasites (as determined by the method of Stauber et al. [22]) in the liver was reduced by 98% when licochalcone A was administered intraperitoneally at a dose of 20 mg/kg of body weight per day for 6 days ($P < 0.05$; Fig. 3). Similarly, the numbers of parasites in the spleens of the same animals were reduced by 96% as determined by the method of Stauber et al. (22) and by 99% as determined by the [^{3}H]thymidine uptake method ($P < 0.05$; Fig. 4).

The results of the effect of oral administration of synthetic licochalcone A to hamsters infected with *L. donovani* are shown in Fig. 5 and 6. Licochalcone A at all three concentrations (5, 50, and 150 mg/kg of body weight) administered for 6 days reduced the parasite loads in the liver by at least 65% ($P < 0.05$; Fig. 5) and in the spleen by more than 70% ($P < 0.05$; Fig. 6).

DISCUSSION

Previously we have demonstrated that licochalcone A, an oxygenated chalcone, inhibits the in vitro growth of the extracellular promastigotes and the intracellular amastigotes of *L. major* and *L. donovani* (5). Here we present data demonstrating that licochalcone A can be used for the control of in vivo infection by both *L. major* in mice and *L. donovani* in hamsters. Licochalcone A inhibited lesion development in the footpads of mice infected with *L. major* and resulted in more than 95% reduction of parasite load in the livers and spleens of hamsters infected with *L. donovani*.

Intraperitoneal administration of licochalcone A at doses of 2.5 and 5 mg/kg of body weight per day completely prevented lesion development in mice infected with *L. major*. Only a

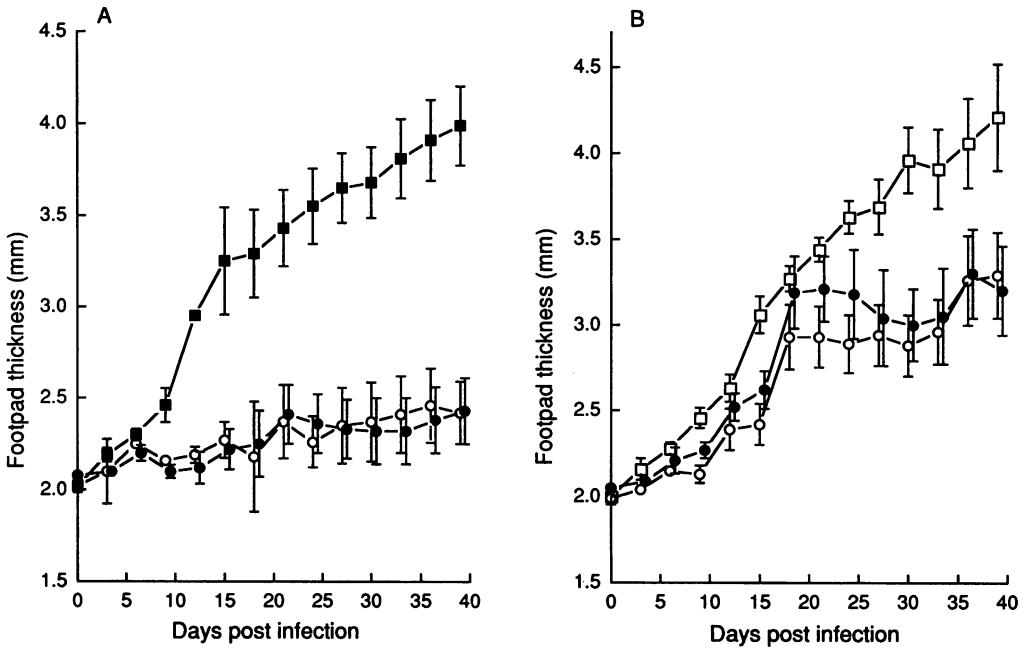

FIG. 1. Effect of licochalcone A on lesion development in BALB/c mice infected in the footpads with 10^7 *L. major* promastigotes. Footpad thicknesses for groups receiving intraperitoneal (A) and intralesional (B) injections of licochalcone A at 2.5 (○) or 5 mg/kg (●) or of buffer (■ or □) are shown. Results are means ± standard errors of the means SEM for 10 mice in each group.

slight infection in the footpads was observed, whereas no parasites could be detected in the spleens and livers of the treated animals. In contrast, intralesional administration of licochalcone A at doses of 1 and 2.5 mg/kg of body weight per day afforded only about 50% reduction in lesion size and slight infections were also observed in the footpads, spleens, and livers of the mice. The better protection by intraperitoneal injection might be due to the inability of the footpad to absorb all the solvents (50 μl) in which licochalcone A was dissolved. This would cause a deposit of solvents in the footpad and an apparent swelling. Another possibility is that at the later stages of footpad infection the lesions were large and hard and some of them cracked, resulting in leakage of licochalcone A.

In addition to protecting mice from infection with *L. major*, licochalcone A also protected hamsters from infection with *L. donovani*. The parasite loads of the livers and spleens of the treated animals were only 2 and 4% of those of controls after intraperitoneal administration of licochalcone A at a dose of 20 mg/kg of body weight per day for 6 consecutive days. When the parasite load in the spleen was estimated by the [^{3}H]thymidine uptake method, an uptake of only 1% of the control values was detected. The advantage of using the [^{3}H]thymidine uptake method is that it measures growth of live parasites, whereas with the Giemsa staining method it might be difficult

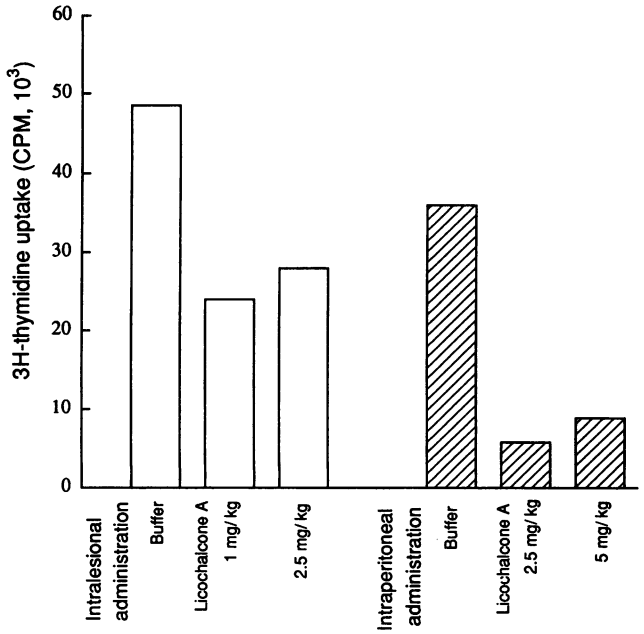

FIG. 2. Effect of licochalcone A given either intraperitoneally or intralesionally on the parasite loads in the footpads of mice infected with *L. major*. Results are mean [^{3}H]thymidine uptake from two experiments in each group.

TABLE 1. Effect of licochalcone A on the numbers of amastigotes in the footpads, spleens, and livers of mice infected with *L. major*

Treatment group	Score for indicated site[a]		
	Footpad	Spleen	Liver
Buffer			
Intraperitoneal	+++	++	++
Intralesional	+++	++	+
Licochalcone A			
1 mg/kg, intralesional	+	+	+
2.5 mg/kg, intralesional	+	+	+
2.5 mg/kg, intraperitoneal	+	−	−
5 mg/kg, intraperitoneal	+	−	−

[a] Results represent the mean numbers of amastigotes per impression smear for two mice in each group. The numbers of amastigotes in five fields are indicated as follows: −, 0; +, <100; ++, 100 to 500; +++, 500 to 1,000.

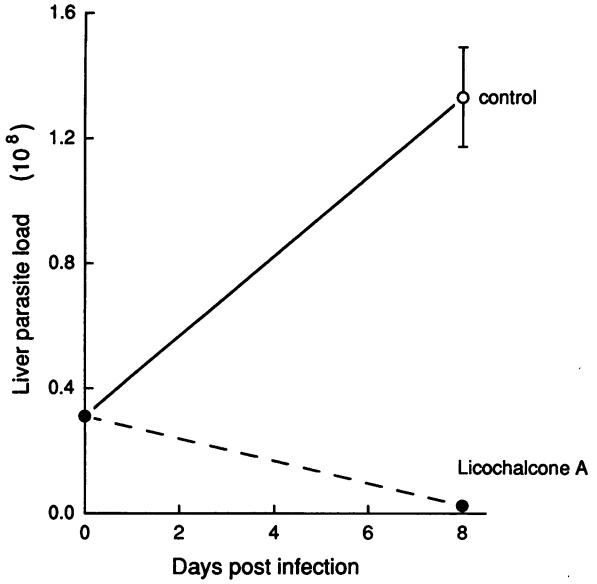

FIG. 3. Effect of licochalcone A given intraperitoneally (10 mg/kg, twice a day for 6 days) on the parasite loads (measured by the method of Stauber et al. [22]) in the livers of hamsters infected with *L. donovani*. Results are means ± SEM for five hamsters in each group.

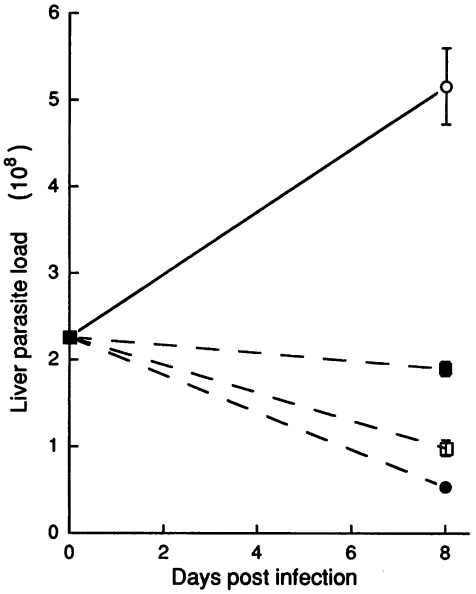

FIG. 5. Effect of licochalcone A on the parasite loads in the livers of hamsters infected with *L. donovani*. Licochalcone A was given orally at 150 (●), 50 (□), and 5 (■) mg/kg once a day for 6 days. Control hamsters received 1% CMC (○) once a day for 6 days. Parasite loads were measured by the method of Stauber et al. (22). Results are means ± SEM for five hamsters in each group.

to distinguish live from dead parasites. Licochalcone A administered orally was also capable of reducing the parasite loads in the spleens and livers of the infected hamsters in a dose-dependent manner. However, the concentrations of the compound required to reduce the parasite load were higher than those given intraperitoneally. One explanation for this difference is that in the oral administration experiment licochalcone A was suspended in 1% CMC solution, whereas in the intraperitoneal administration experiment it was dissolved in dimethyl sulfoxide. CMC solution is a nontoxic standard carrier for toxicity studies. Licochalcone A exhibits a low level of solubility in CMC solution and in water. The low water solubility of licochalcone A might result in absorption of

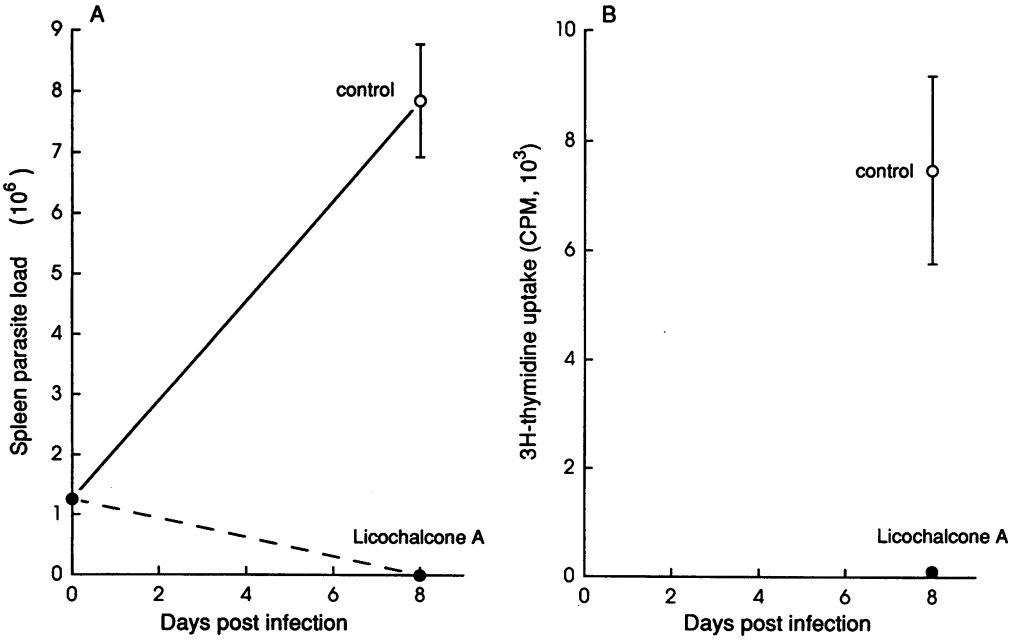

FIG. 4. Effect of licochalcone A given intraperitoneally (10 mg/kg, twice a day for 6 days) on the parasite loads in the spleens of hamsters infected with *L. donovani*. Parasite loads were measured by the method of Stauber et al. (22) (A) and by [^{3}H]thymidine uptake (B). Results are means ± SEM for five hamsters in each group.

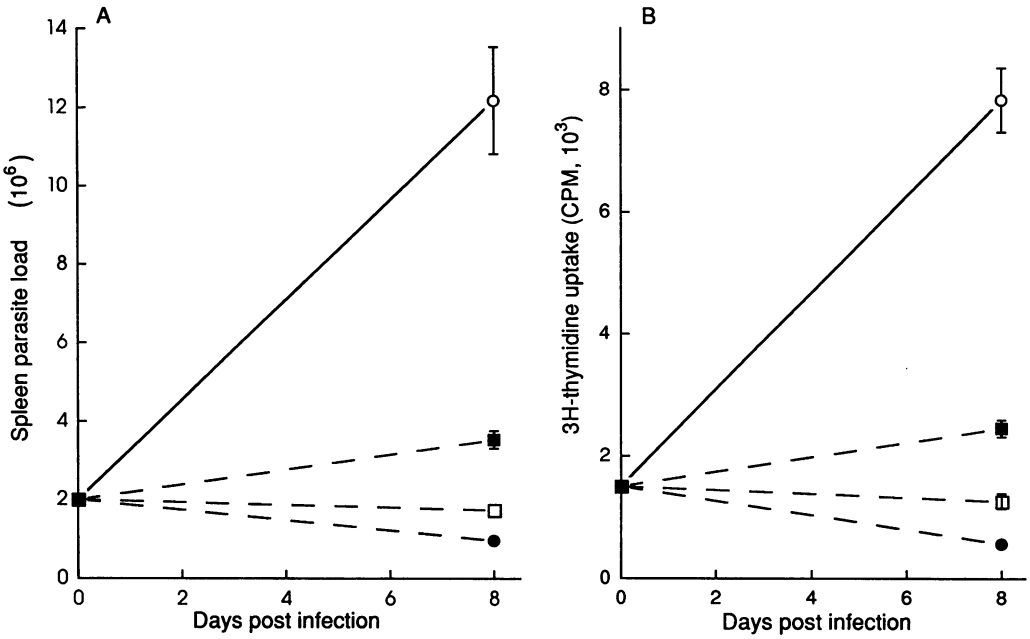

FIG. 6. Effect of licochalcone A on the parasite loads in the spleens of hamsters infected with *L. donovani*. Licochalcone A was given orally at 150 (●), 50 (□), and 5 (■) mg/kg once a day for 6 days. Control hamsters received 1% CMC (○) once a day for 6 days. Parasite loads were measured by the method of Stauber et al. (22) (A) and by [^{3}H]thymidine uptake (B). Results are means ± SEM for five hamsters in each group.

smaller amounts of the compound through the gastrointestinal tract. Studies to find appropriate nontoxic solvents to improve the solubility of licochalcone A are in progress.

Preliminary experiments were carried out to examine the potential toxicity of licochalcone A in vivo. Licochalcone A at concentrations of up to 1,000 mg/kg in 1% CMC, administered orally to rats once a day for 2 weeks, did not exhibit any observable signs of toxicity in these animals. Intraperitoneal injections of dimethyl sulfoxide-dissolved licochalcone A at concentrations of 100 mg/kg in rats and 150 mg/kg in hamsters did not show any toxicity.

The mechanism by which licochalcone A protects the animals from *L. major* and *L. donovani* infections is not known. However, our previous study indicates that the target organelle for licochalcone A appears to be the parasite mitochondria (5). Further studies to elucidate the exact mechanism of action of licochalcone A against *Leishmania* parasites are warranted.

For studies on the search for antileishmanial drugs, other groups have used the hamster-*L. donovani* model. Kinnamonn et al. have examined a series of lepidines (8-aminoquinoline derivatives) and have shown that when given orally one of these derivatives, designated WR 6026, was over 700 times as effective as the conventional antimonial drugs (11). Berman et al. have shown that liposome-encapsulated amphotericin B at a total dose of 1.5 mg/kg eliminated more than 99% of the hepatosplenic parasites in *L. donovani*-infected hamsters (4). Stauber et al. (22) reported that pentamidine reduced the parasite loads of the livers of hamsters infected with *L. donovani* to approximately 31% of the control value at a dose of 12.5 mg/kg of body weight and that pentostam at doses of 47 and 94 mg/kg of body weight reduced the liver parasite loads to approximately 20 and 2% of control values. Ghosh et al. (8) reported that berberine reduced the liver parasite load to 51% of control levels at a dose of 50 mg/kg of body weight.

Pentavalent antimony compounds have been in use for the last 40 years for the treatment of various forms of leishmaniasis (3, 9, 10). However, the toxicity, the side effects, and the duration of treatment are major drawbacks (23). Recently, the occurrence of large-scale clinical resistance against the antimonial agents has been reported (24). Therefore, there is a great need for the development of effective and safe drugs for the treatment of leishmaniasis. A number of studies of chemotherapy of leishmaniasis have been carried out during the last two decades (4, 11, 14, 15, 17, 19, 25). These studies indicate that amphotericin B, liposomal amphotericin B, and paromomycin are more active than the antimonial agents for visceral leishmaniasis and pentamidine for cutaneous leishmaniasis and that allopurinol is being clinically tested in treatment of cutaneous leishmaniasis (15). The results presented in this study show that licochalcone A protected mice and hamsters from infections with *L. major* and *L. donovani*, the causative agents of cutaneous and visceral leishmaniasis, respectively. Of major importance is the activity of licochalcone A against *L. donovani* when administered orally. Our previous in vitro results (5) together with the in vivo data presented here clearly establish that licochalcone A might provide the lead for the development of a new class of antileishmanial drugs. Studies to examine the effect of various analogs of licochalcone A on *Leishmania* parasites and to elucidate the structure-activity relationship of these compounds are in progress at our laboratory.

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