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Licochalcone A, a Novel Antiparasitic Agent with Potent Activity against Human Pathogenic Protozoan Species of *Leishmania*

MING CHEN,[1,2] SØREN BRØGGER CHRISTENSEN,[3] JENS BLOM,[4] ELSE LEMMICH,[3] LYNDA NADELMANN,[3] KAREN FICH,[3] THOR G. THEANDER,[5,6] AND ARSALAN KHARAZMI[1]*

Department of Clinical Microbiology, Centre for Medical Parasitology,[1] and Department of Infectious Diseases,[5] University Hospital, and Laboratory of Molecular Cell Biology,[4] Statens Seruminstitut, Department of Organic Chemistry, The Royal Danish School of Pharmacy,[3] and Institute for Medical Microbiology and Immunology, Copenhagen University,[6] Copenhagen, Denmark, and Department of Pathophysiology, Guangxi Medical College, Nanning, China[2]

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Licochalcone A, an oxygenated chalcone isolated from the roots of Chinese licorice plant, inhibited the growth of both *Leishmania major* and *Leishmania donovani* promastigotes and amastigotes. The structure of the licochalcone A was established by mass and nuclear magnetic resonance spectroscopies and by synthesis, and its purity was verified by high-pressure liquid chromatography. The 50% inhibition of growth of logarithmic- and stationary-phase promastigotes of *L. major*, as measured by [^{3}H]thymidine uptake, were 4 and 2.5 μg/ml, respectively. The growth of *L. major* promastigotes was totally inhibited after a 20-h incubation period with licochalcone A at 5 μg/ml. At a concentration of 0.5 μg/ml, licochalcone A markedly reduced the infection rate of human peripheral blood monocyte-derived macrophages and U937 cells with *L. major* promastigotes and exhibited a strong intracellular killing of the parasite. These data show that intracellular *Leishmania* amastigotes are more susceptible than promastigotes to licochalcone A. Results of studies on the site of action of licochalcone A indicate that the target organelle appears to be the parasite mitochondria. These findings demonstrate that licochalcone A in concentrations that are nontoxic to host cells exhibits a strong antileishmanial activity and that appropriate substituted chalcones might be a new class of antileishmanial drugs.

Leishmaniases are a broad spectrum of diseases caused by different species of protozoan parasites of the genus *Leishmania*. The diseases are prevalent in many parts of the world, particularly Africa, Asia, and Latin America (17). The diseases are also endemic in many parts of southern Europe. Some 350 million people are at risk of infection with *Leishmania* species, and more than 12 million people are infected with different species of the parasite, with over 400,000 new cases annually (1). Recently, a dramatic increase in the number of visceral cases has been observed in Europe. This disease occurs in Europe in its fulminant form in patients with compromised T-cell function such as in those with human immunodeficiency virus infections (patients with AIDS), patients with malignancies, and patients undergoing immunosuppressive therapy (2, 9, 11). The drugs available for the treatment of leishmaniases were developed before 1960 (21) and are, in general, toxic and expensive, and treatment with these drugs is long term. Large-scale clinical resistance against the most commonly used antileishmanial drug, the antimonial agents, has been reported (37). A serious epidemic of visceral leishmaniasis is ravaging southern Sudan, and recent reports estimate that over 60,000 deaths have occurred during the past 5 years and more than 25% of the local population is infected with kala azar (visceral leishmaniasis). There is a risk of the disease spreading into other areas of Africa, with devastating consequences for the population. Several *L. donovani* isolates from patients with visceral leishmaniasis in Sudan have been found to be resistant to the antimonial drug Pentostam (unpublished data from our group). The spread of drug resistance combined with other shortcomings of the available antileishmanial drugs emphasizes the importance of the development of new, effective, and safe drugs against leishmaniases.

The discovery of new drugs from traditional medicine is not a new phenomenon. Worldwide, more than 100 clinically useful prescription drugs are derived from higher plants. About 74% of these came to the attention of pharmaceutical companies because of their use in traditional medicine (34). There are reports on the potential of plants as sources of new antiprotozoal agents (28, 29). Licorice has been used in China for the treatment of gastric and duodenal ulcers, bronchial asthma, Addison's disease, food and drug poisoning, and skin diseases such as eczema and urticaria (35).

The present study was designed to examine the potential antileishmanial activity of extracts of Chinese licorice roots. We found that an alcohol extract from the licorice roots inhibited the growth of *L. major* and *L. donovani* promastigotes. Following this finding, the compound with antileishmanial activity was purified from the plant by a bioassay-guided chemical fractionation of the extract. In this report, we show that licochalcone A, an oxygenated chalcone purified from the roots of Chinese licorice plant, exhibits potent antileishmanial activity.

MATERIALS AND METHODS

Plant extract. The extraction procedure was a modification of the method described by Ngam et al. (22). Briefly, 20 g of dried roots of licorice obtained from the Xinjiang Province of China were cut into small pieces, soaked in 350 ml of distilled water at room temperature overnight, and then

* Corresponding author.

boiled for 90 min. The extract was decanted and filtered from the plant residue. The crude aqueous extract was mixed with 96% ethanol in a ratio of 1:4 (vol/vol). A gel-like precipitate formed immediately. The alcohol extract mixture was centrifuged at 5,900 × *g* for 30 min at 20°C. The supernatant was sterile filtered through a 0.22-μm-pore-size Millipore filter. The filtered supernatant was evaporated to dryness. The dried material was dissolved in a 0.9% NaCl solution for use in the parasite growth inhibition assays. The final undiluted extract solution contained 1.25 g of dried plant material equivalent per ml.

Fractionation. The promastigote growth inhibition assay was used as a guide to identify fractions exhibiting antileishmanial activity. The residue of an alcoholic extract of 674 g of licorice roots was partitioned between water and ethyl acetate. Only the organic phase showed antiparasitic activity. The residual gum from the organic phase was partitioned between hexane and methanol-water (9:1). The residue of the methanolic phase was rechromatographed over silica gel by using toluene-ethyl acetate (9:1), to which increasing amounts of ethyl acetate were added as the eluent. The residues of the fractions possessing the major activity were rechromatographed over silica gel by using eluents comprising methylene chloride-ethyl acetate (14:1), to which increasing amounts of ethyl acetate were added. The residues of the fractions possessing the major activity (0.6 g) were crystallized from methanol-water to give 0.3 g of reddish crystals.

Structure elucidation. The structure of licochalcone A was established by mass and nuclear magnetic resonance spectroscopies and by synthesis. Comparison of the nuclear magnetic resonance and mass spectroscopic data for the isolated chalcone with the published data (12, 24, 33) established the identity of the compound as licochalcone A. The melting point of the licochalcone A crystals was determined to be 101 to 102°C (33). Further proof of the identity was obtained by total synthesis of the compound by published procedures (13, 32). The purity of the compound was established by high-pressure liquid chromatography by using a LiChrosorb RP 18 column and a mobile phase consisting of acetonitrile-aqueous acetic acid (2%) (1:1) as an eluent.

Parasite cultures. A World Health Organization reference vaccine strain of *L. major*, originally isolated from a patient in Iran and kindly provided by R. Behin, Immunology Research and Training Centre, World Health Organization, Lausanne, Switzerland, and two Kenyan strains of *L. donovani* (MHOM/KE/85/NLB 274 and MHOM/KE/85/NLB 439), kindly provided by the Kenya Medical Research Institute, Nairobi, were used. Promastigotes were cultured at 26°C in RPMI 1640 containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 4 mM L-glutamine, 0.02 mg of gentamicin per ml, and 10% heat-inactivated fetal calf serum.

Effect on promastigotes. The effects of licorice root extract and licochalcone A on promastigotes were assessed by a method similar to the one described by Pearson et al. (26). Promastigotes were incubated at 26°C in the presence of twofold serial dilutions of the extract, different concentrations of licochalcone A or the medium alone in 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark). After 2 h, 1 μCi of [^{3}H]thymidine (New England Nuclear, Boston, Mass.) was added to each well. Parasites were harvested 18 h later, and [^{3}H]thymidine incorporation was measured. All cultures were performed in triplicate. The promastigotes were also counted and their flagellar motilities were assessed microscopically.

Effect on intracellular amastigotes. The *L. major* promastigotes used in the present study were obtained from the footpad tissues of BALB/c mice infected 1 to 2 months previously by subcutaneous inoculation with 10^7 stationary-phase promastigotes. The promastigotes were passaged in culture two times before use. The effect on amastigotes was examined in infected human peripheral blood monocyte-derived macrophages (MDMs) and U937 cells (16). Two methods were used.

(i) Microscopic counting. Microscopic counting of the amastigotes in the macrophages was done by a modification of the method described by Berman and Wyler (5). One milliliter of a suspension containing 5 × 10^6 human peripheral blood mononuclear cells was added to each well (16 mm in diameter containing one piece of cover glass with a diameter of 12 mm) of a 24-well plastic culture plate (Nunc). After 6 days of culture, 1 ml of 10^7 stationary-phase *L. major* promastigotes per ml were added to each well, and the cultures were then incubated at 34°C. After 24 h, the cultures were washed three times and incubated in the medium containing different concentrations of licochalcone A or the medium alone. At 3 and 6 days after infection, the macrophage cultures were fixed with absolute methanol, stained with 5% Giemsa stain for 10 min, and examined by light microscopy. The percentage of macrophages containing amastigotes and the number of amastigotes per infected macrophage were determined in replicate cultures by counting 500 cells.

(ii) [^{3}H]thymidine incorporation. [^{3}H]thymidine incorporation by the parasite was used to measure intracellular antiparasite activity. This was a modification of a previously described method (6). Human MDMs were prepared, infected with the parasite, and treated with licochalcone A the same way as described above for the microscopic counting method. Three days after infection, macrophages were lysed with 0.01% sodium dodecyl sulfate (SDS) and the cultures were transferred to a 26°C incubator, in which the amastigotes were left to transform into promastigotes. After 48 h, parasite growth was determined by adding 1 μCi of [^{3}H]thymidine to each well. The parasites were harvested 24 h later and [^{3}H]thymidine incorporation was measured.

Ultrastructure studies. Electron microscopic studies were carried out to examine the effect of licochalcone A on the ultrastructure of the parasite. A suspension of 3 × 10^6 *L. major* promastigotes per ml was incubated in the presence of either 10 μg of licochalcone A per ml or medium alone for 24 h at 26°C. After incubation, the promastigotes were centrifuged at 500 × *g* and were resuspended in 1 ml of medium. They were fixed with glutaraldehyde. After fixation, the

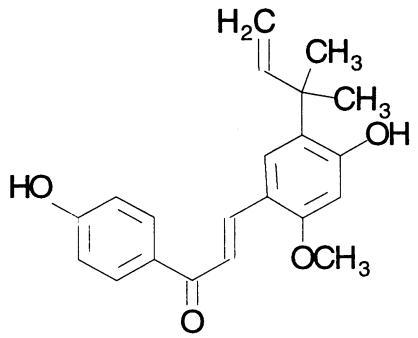

FIG. 1. Chemical structure of licochalcone A.

TABLE 1. Effect of licorice root extract on the growth of *L. major* and *L. donovani* promastigotes and the flagellar motility and morphology of promastigotes from 3-day cultures

Dilution	% of control[a]		% of parasites[b]	
	L. major	*L. donovani*	Flagellar motility	Abnormal round morphology
1:100	0.4 ± 0.2[c]	1 ± 0.6[c]	0.5 ± 0.7[c]	92 ± 7[c]
1:200	3 ± 2[c]	11 ± 8[c]	14 ± 13[c]	74 ± 13[c]
1:400	16 ± 9[d]	42 ± 8[d]	25 ± 14[d]	48 ± 18[d]
1:800	41 ± 15[d]	68 ± 7[e]	61 ± 4[e]	31 ± 6[d]
1:1,600	71 ± 9[e]	82 ± 4	78 ± 9[e]	24 ± 7[e]
1:3,200	85 ± 14	90 ± 9	84 ± 9	13 ± 9
Medium alone			100	8 ± 6

[a] The results of the in vitro growth of promastigotes are from three experiments and are given as mean ± standard error of the mean percentages of control [mean counts in control promastigotes were (59 ± 3) × 10^3 cpm]).

[b] The flagellar motilities and morphologies of *L. donovani* promastigotes were determined microscopically by examination of 500 promastigotes. The results are given as mean ± standard error of the mean percentages of parasites from four experiments.

[c] Values significantly lower than control value ($P < 0.0001$).

[d] Values significantly lower than control value ($P < 0.01$).

[e] Values significantly lower than control value ($P < 0.05$).

parasites were centrifuged at 8,800 × *g* for 2 min and embedded in warm (45°C) 1.5% Noble agar (Difco). Agar blocks containing parasites were cut into small cubes of 1 mm^3 and postfixed for 90 min in 1% OsO_4 in 0.1 M cacodylate buffer. The blocks were stained in 2% uranyl acetate in barbiturate buffer and, after dehydration, were embedded in Vestopal W (Martin Jaeger, Geneva, Switzerland). The sections were poststained with magnesium uranyl acetate and lead citrate and were examined in a Philips 201 C electron microscope.

Lymphocyte proliferation. The proliferation of human peripheral blood lymphocytes was determined as described previously (7).

Chemiluminescence. A luminol-enhanced chemiluminescence assay was used to determine the oxidative burst response of human peripheral blood neutrophils and monocytes (14).

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

RESULTS

The chemical structure of licochalcone A is shown in Fig. 1. Licochalcone A belongs to the chalcones, which are characterized by being α,β-unsaturated bisphenylic ketones. In licochalcone A, the chalcone skeleton is substituted with two phenolic hydroxyl groups, one methoxy group, and an isoprenoid side chain. The α,β-unsaturated ketone group enables the chalcone to alkylate *N*-acetyl-L-cysteine and other biomolecules possessing thiolo groups, and the phenolic hydroxy groups might function as free oxygen-radical scavengers.

The dose-dependent antiparasitic effect of the extract on *L. major* and *L. donovani* is shown in Table 1. A significant reduction in the growth of promastigotes of *L. major* and *L. donovani* in logarithmic-phase cultures was observed at an extract dilution of 1:800 ($P < 0.05$). The 50% inhibition of the logarithmic-phase promastigotes was at extract dilutions of between 1:400 and 1:800. At an extract dilution of 1:100, total inhibition of promastigote growth occurred. More than 90% inhibition of promastigote growth was observed with extract dilutions of between 1:100 and 1:200. In parallel experiments, an extract from another Chinese plant (*Fructus aristolochiae*) prepared exactly the same way that the extract from the licorice roots was prepared did not exhibit any inhibitory effect on *L. major* and *L. donovani* promastigotes at the lowest dilution tested (1:100) (data not shown). Promastigote viability was also assessed by morphological criteria such as flagellar motility and parasite morphology (Table 1). A 1:200 dilution of the extract inhibited the motilities of more than 85% of the promastigotes. At that dilution, about 75% of the promastigotes exhibited an abnormal round morphology.

Licochalcone A exhibited a strong inhibitory effect on both the 6-day and the 3-day cultures of the parasite (Fig. 2). In both *L. major* and *L. donovani*, the concentrations that inhibited 50% growth of the logarithmic- and stationary-phase promastigotes were 2.5 to 4 μg/ml, respectively. As shown in Fig. 3, when the parasites were incubated with licochalcone A for a longer period of time, the inhibitory

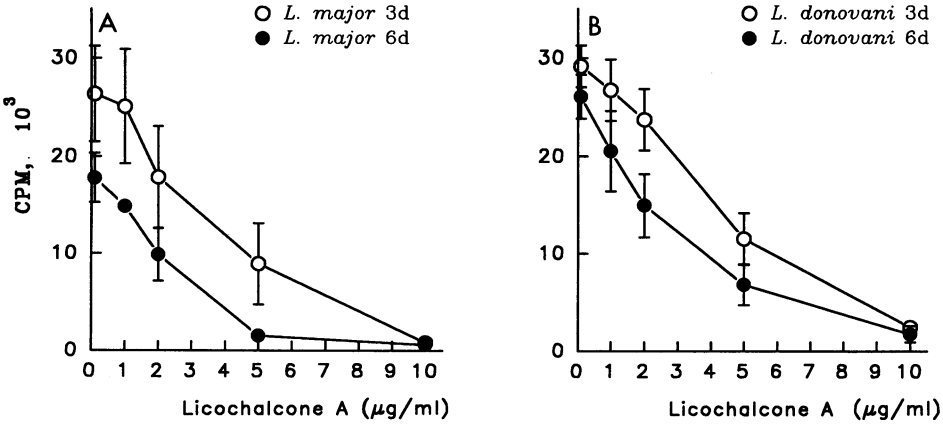

FIG. 2. Effect of licochalcone A on the growth of *L. major* (A) and *L. donovani* (B) promastigotes from 3- and 6-day cultures. A total of 3 × 10^6 promastigotes were incubated in the presence of 1, 2, 5, and 10 μg of licochalcone A per ml for 2 h; this was followed by 18 h of incubation in the presence of [^{3}H]thymidine. The results are from five experiments and are given as percentage of control (mean ± standard error of the mean), as measured by [^{3}H]thymidine incorporation.

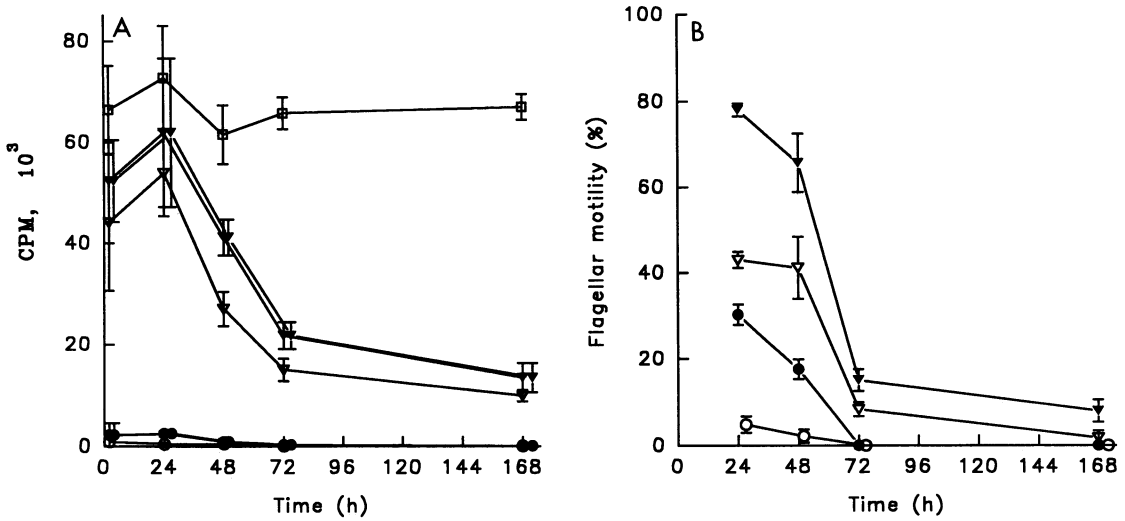

FIG. 3. Time study on the effect of licochalcone A on the in vitro growth of *L. major* promastigotes from 4-day cultures. The results are from five experiments and are given as 10^3 counts per minute (mean ± standard error of the mean), as measured by [^{3}H]thymidine incorporation (A), and flagellar motility (mean ± standard error of the mean) of parasites, as determined microscopically by counting 500 promastigotes (B). Symbols: ○, 10 μg/ml; ●, 5 μg/ml; ▽, 1 μg/ml; ▼, 0.5 μg/ml; □, control.

activity was stronger and the dose of licochalcone A required was much less.

Table 2 shows that licochalcone A markedly inhibited the in vitro growth of *L. major* amastigotes in human MDMs and U937 cells. No amastigotes were seen in macrophages incubated with 5 and 10 μg of licochalcone A per ml. A significant reduction in the number of amastigotes was observed at 1 and 0.5 μg of licochalcone A per ml ($P < 0.05$). Using the [^{3}H]thymidine incorporation assay to measure the intracellular parasite killing activity of licochalcone A, it was shown that the parasite growth in both human MDMs and U937 cells was decreased by more than 95% with 1 μg of licochalcone A per ml (Fig. 4).

Electron microscopic studies were carried out to determine the possible ultrastructural changes of the parasites when they were incubated with licochalcone A. Figure 5 shows electron micrographs of promastigotes incubated in the presence of 10 μg of licochalcone A per ml (Fig. 5A) or medium alone (Fig. 5C). Figure 5A shows a section through four promastigotes incubated with 10 μg of licochalcone A per ml, showing greatly enlarged empty, light mitochondria. Other organelles appeared to be normal. The kinetoplast was seen in two of the affected mitochondria. Figure 5B shows mitochondria cristae (arrowheads) in one of the affected mitochondria (arrow in Fig. 5A). Figure 5C shows the section through promastigotes incubated with medium

TABLE 2. Effect of licochalcone A on *L. major* amastigotes in human MDMs and U937 cells[a]

Day after infection	Liochalcone A concn (μg/ml)	MDMs		U937	
		% of infected cells	No. of Amastigotes/ infected cell	% of infected cells	No. of amastigotes/ infected cell
0		36 ± 1	2.2 ± 0.1	41 ± 1	3.0 ± 0.1
3	10	0 ± 0[b]		0 ± 0[b]	
	5	0 ± 0[b]		0 ± 0[b]	
	1	11 ± 1[b]	1.6 ± 0.1	19 ± 1[b]	2.0 ± 0.1[b]
	0.5	14 ± 1[b]	1.7 ± 0.2[c]	28 ± 1[b]	2.3 ± 0.1[c]
	0	44 ± 1	4 ± 0.2	43 ± 1	5.7 ± 0.2
6	10	0 ± 0[b]		0 ± 0[b]	
	5	0 ± 0[b]		0 ± 0[b]	
	1	7 ± 0[b]	2.2 ± 0.1[c]	3 ± 1[b]	1.8 ± 0.1[b]
	0.5	8 ± 1[b]	2.0 ± 0.1[c]	14 ± 1[b]	3.0 ± 0.1[b]
	0	44 ± 1	4.4 ± 0.1	47 ± 1	6.3 ± 0.1

[a] Data are given as mean ± standard error of the mean percentages of experiments.
[b] Values significantly lower than control value ($P < 0.001$).
[c] Values significantly lower than control value ($P < 0.05$).

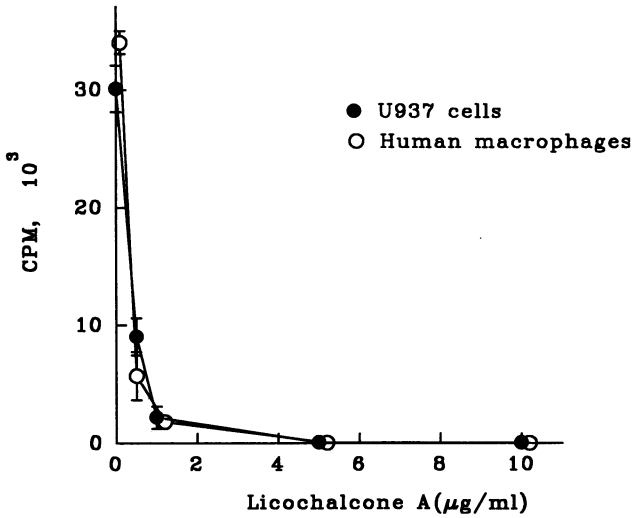

FIG. 4. Effect of licochalcone A on intracellular parasite killing. Human MDMs and U937 cells infected with *L. major* promastigotes were incubated in the presence of licochalcone A for 3 days; this was followed by SDS lysis and further incubation. The proliferation of surviving parasites was measured by [^{3}H]thymidine incorporation. The results are from five experiments and are given as 10^3 counts per minute (mean ± standard error of the mean).

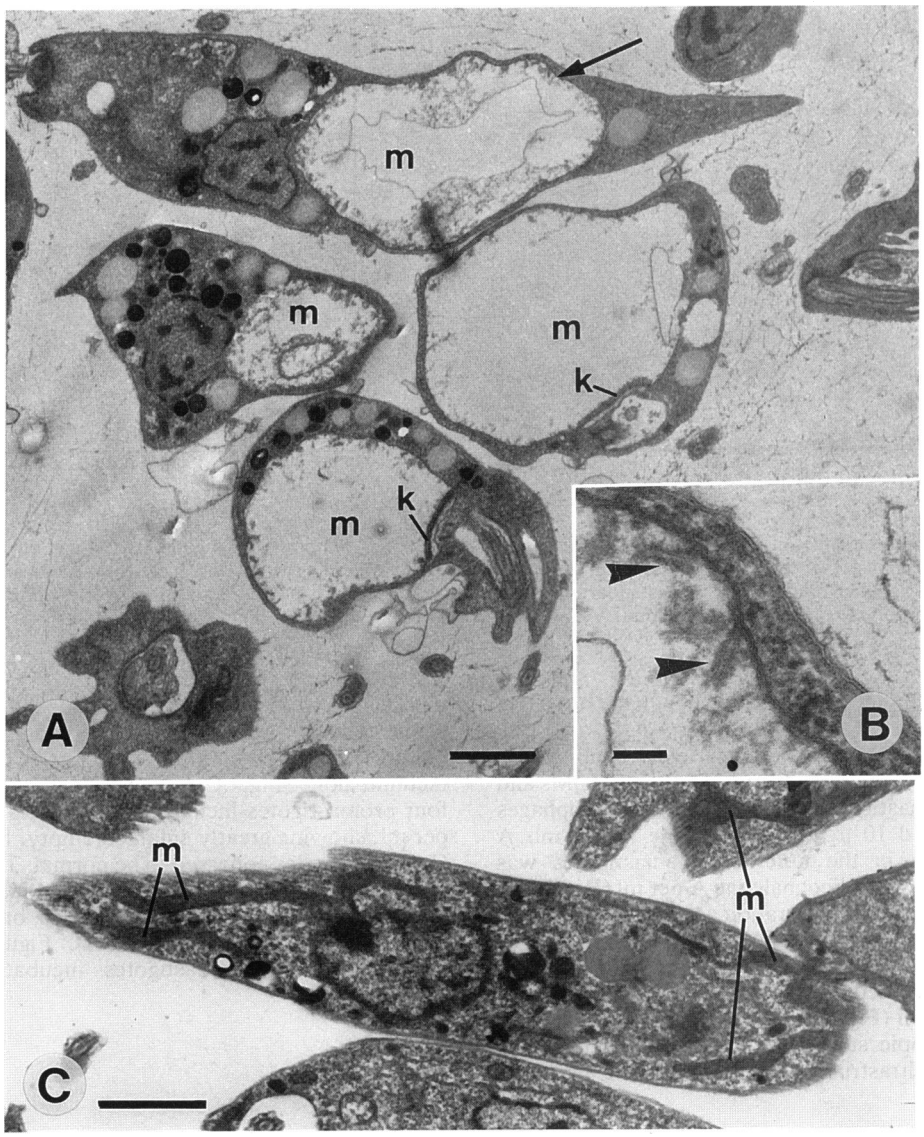

FIG. 5. Electron micrographs of promastigotes incubated in the presence of 10 μg of licochalcone A per ml (A) or medium alone (C). (A) A section through four promastigotes incubated with 10 μg of licochalcone A per ml showing greatly enlarged empty, light mitochondria (m). The kinetoplast (k) is seen in two of the affected mitochondria. Magnification, ×11,100. Bar, 1 μm. (B) Mitochondria cristae (arrowheads) in one of the affected mitochondria (arrow in panel A). Magnification, ×68,200. Bar, 0.1 μm. (C) Section through promastigotes incubated with medium alone, showing long, slender, dense, dark, and normal-looking mitochondria (m). Magnification, ×13,400. Bar, 1 μm.

alone, showing long, slender, dense, dark, and normal-looking mitochondria.

Further experiments were carried out to determine the potential toxicity of licochalcone A on human leukocytes. It was shown that licochalcone A at a concentration of 10 μg/ml inhibited the proliferative response of human lymphocytes to phytohemagglutinin by about 40% and the chemiluminescence response of human neutrophils and monocytes to opsonized zymosan by about 30%. There were little or no inhibitory effects at lower concentrations.

DISCUSSION

The data presented in this report demonstrate that an alcoholic extract of licorice roots containing licochalcone A as the major active principle exhibits strong antileishmanial activity. Licochalcone A is one of the many flavonoids which have been found to be present in the Chinese drug Gan Cao (31). Three species of licorice plants, *Glycyrrhiza glabra*, *Glycyrrhiza uralensis*, and *Glycyrrhiza inflata*, are accepted by the Chinese pharmacopoeia as sources for this drug (31). The amount of licochalcone A present in the three species has, however, been found to vary considerably (31, 39). The structure of licochalcone A was first reported in 1975, and the compound was found to be an example of the very rare retrochalcones (33). Other studies have revealed that licochalcone A exhibits antimicrobial and antioxidant activities (24) and that it inhibits calcium ionophore-induced leukotriene B_4 and leukotriene C_4 formation of human polymorphonuclear neutrophils (15). In addition, licochalcone A has been reported to possess anti-inflammatory and anti-tumor-promoting activities (36). Here we reported on a novel

antiparasitic activity of licochalcone A, which makes it a promising lead for the development of much-needed antileishmanial drugs.

Our project was initiated by the observation that the extract of licorice roots inhibited the in vitro growth of both *L. major* and *L. donovani* promastigotes (Table 1). Following bioassay-guided fractionation, licochalcone A was identified as the major antileishmanial element in the Chinese licorice roots. Licochalcone A was shown to inhibit the in vitro growth of *L. major* and *L. donovani* promastigotes at both the logarithmic and the stationary stages. The [^{3}H]thymidine uptake of *L. major* promastigotes was totally inhibited after 20 h of incubation in the presence of 5 μg of licochalcone A per ml, indicating interference with DNA synthesis in the parasites.

Licochalcone A reduced the infection rate of human MDMs and U937 cells with *L. major* promastigotes and exhibited a remarkably strong inhibition of intracellular parasite multiplication. The concentration of licochalcone A required for inhibitory activity against amastigotes was much lower that required for inhibitory activity against promastigotes, indicating that the intracellular amastigote form of *Leishmania* parasites is more susceptible to licochalcone A than the extracellular promastigote form is.

The studies on the ultrastructures of the parasites showed that licochalcone A destroyed the mitochondria. There were no apparent changes in the other organelles of the parasite. On the other hand, the mitochondria of macrophages incubated with similar concentrations of licochalcone A were not affected by this compound (data not shown), indicating the specificity of licochalcone A for parasite mitochondria. The reason for this specificity is not clear at present. The study on the effect of licochalcone A on the functions of the cells of the human immune system showed that licochalcone A, at the concentrations which exhibited potent antileishmanial activity, did not exhibit major toxicity to human lymphocytes, polymorphonuclear leukocytes, or monocytes.

Treatment of patients with leishmaniasis still poses a serious problem. Most of the commonly used antileishmanial drugs, such as pentavalent antimonial agents (SbV), exhibit considerable toxicity, and there are reports of large-scale clinical drug resistance among the organisms that cause visceral leishmaniasis (37). Second-line drugs, such as pentamidine and amphotericin B, do not have a therapeutic index as favorable as that of SbV, long-term therapy is often required, and they often induce toxic effects (4, 26). A number of investigations to explore potential antileishmanial drugs have been carried out during the last decade (3, 5, 8, 10, 18, 19, 22, 25–27, 30, 38). It has been reported that chlorpromazine (26), methylbenzethonium chloride (10), inosine analogs (19), sulfonamides (27), pyrimidines (18, 30), allopurinol (18, 20), trifluralin (8), and berberine derivatives (38) have antileishmanial activities. However, so far only one of these compounds, allopurinol, has been developed for clinical study (20).

The major discovery presented in this report is that licochalcone A exhibits potent activity against *L. donovani*, the cause of fatal visceral leishmaniasis. The importance of this finding is emphasized by the fact that this disease is an alarming health problem because of the development of resistance against the antimonial agents, the most commonly used antileishmanial drugs. It is crucial for any drug against this disease to inhibit growth and multiplication of the amastigote form of the parasite, since *Leishmania* parasites exist solely inside macrophages during established infections. Licochalcone A fulfills this requirement since it potently inhibits growth of the amastigote (Table 2). In addition, licochalcone A also inhibits the infective promastigote form of the parasite, and has therefore the potential of preventing macrophage infection in a prophylactic manner. The pronounced changes in the ultrastructures of the mitochondria of parasites treated with licochalcone A indicates that the energy metabolism of the parasites is affected. In contrast, fairly high concentrations of the compound do not appear to exhibit any toxicity against human phagocytic cells. These findings, together with the fact that licochalcone A has been indicated for use for food conservation and the worldwide use of licorice in medicine, point to the safety of the potential drug for use in humans.

Experiments are in progress at our laboratory to test the antileishmanial activity of licochalcone A in in vivo animal models and the possible toxicity of this compound in animals. Furthermore, studies are in progress to examine the effects of various analogs of licochalcone A on *Leishmania* parasites and to elucidate the structure-activity relationships of these compounds.

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