The Antileishmanial Agent Licochalcone A Interferes with the Function of Parasite Mitochondria

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Leishmaniasis is a major and increasing public health problem, particularly in Africa, Asia, and Latin America (18, 20), and it consists of a broad spectrum of diseases caused by different species of the protozoan genus *Leishmania*. 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, and there are over 400,000 new cases each year (1). Recently, a dramatic increase in the number of visceral cases has been observed in southern Europe. The drugs available for the treatment of leishmaniasis are, in general, toxic and expensive and require long-term treatment (18). Large-scale clinical resistance against the most commonly used antileishmanial drug, antimonials, has been reported (19). 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 leishmaniasis.

Our previous studies have shown that licochalcone A, an oxygenated chalcone, has antileishmanial (M. Chen, S. B. Christensen, J. Blom, E. Lemmich, L. Nadelmann, K. Fich, T. G. Theander, and A. Kharazmi, Antimicrob. Agents Chemother. 37:2550–2556, 1993; M. Chen, S. B. Christensen, T. G. Theander, and A. Kharazmi, Antimicrob. Agents Chemother. 38:1339–1344, 1994) and antimalarial (M. Chen, T. G. Theander, S. B. Christensen, L. Hviid, L. Zhai, and A. Kharazmi, Antimicrob. Agents Chemother. 38:1470–1475, 1994) activities. We have observed that licochalcone A alters the ultrastructure of the mitochondria of *Leishmania* promastigotes (Chen et al., Antimicrob. Agents Chemother. 37:2550–2556, 1993). The present study was designed to examine this observation further and investigate the mechanism of action of antileishmanial activity of licochalcone A. Electron microscopic studies showed that licochalcone A altered the ultrastructure of *Leishmania major* promastigote and amastigote mitochondria in a concentration-dependent manner without damaging the organelles of macrophages or the phagocytic function of these cells. Studies on the function of the parasite mitochondria showed that licochalcone A inhibited the respiration of the parasite in a concentration-dependent manner, as shown by inhibition of O2 consumption and CO2 production by the parasites. Moreover, licochalcone A inhibited the activity of the parasite mitochondrial dehydrogenase. The inhibition of the activity of the parasite mitochondrial enzyme correlated well with the changes in the ultrastructure of the mitochondria shown by electron microscopy. These findings demonstrate that licochalcone A alters the ultrastructure and function of the mitochondria of *Leishmania* parasites.

MATERIALS AND METHODS

Licochalcone A. Licochalcone A was synthesized in our laboratory as previously described (6, 7), and it was dissolved in 2% dimethyl sulfoxide in medium 199 to prepare a working solution of 1 mg/ml.

Parasite cultures. A World Health Organization reference vaccine strain of *L. major* (MHOM/IL/67/LRC-L437) originally isolated from a patient in Iran was cultured in medium 199 containing 10% heat-inactivated fetal calf serum. Incubation and growth of the parasites were carried out at 26°C. Promastigotes were harvested on culture day 4 and used.

Ultrastructure studies. Electron microscopic studies were carried out to examine the effect of licochalcone A on the ultrastructure of both the promastigote and amastigote forms of the parasite as previously described (5). Briefly, for the promastigote study, a suspension of 3 × 10⁶ *L. major* promastigotes per ml was incubated in the presence of either licochalcone A or medium alone for 24 h at 26°C. After incubation, the promastigotes were centrifuged, resuspended in 1 ml of medium, fixed with 3% glutaraldehyde, and embedded in Noble agar. After postfixation in OsO₄, blocks were stained in 2% uranyl acetate and embedded in Vestopal W. The sections were poststained with magnesium uranyl acetate and lead citrate and examined in a Philips 201C electron microscope at 60 kV. For the amastigote study, human peripheral blood monocyte-derived macrophages (MDM) were infected with *L. major* promastigotes as described previously (5) and then incubated with licochalcone A for 72 h at 37°C. An uninfected macrophage culture treated with licochalcone A was used as a control. The macrophages were removed from the cultures by treatment with trypsin, centrifuged at...
500 \times 10^3 g$, and then resuspended in 1 ml of medium. The rest of the steps were the same as in the promastigote study.

**Monocyte chemiluminescence and viability.** A lumino-enhanced chemiluminescence assay was used to determine the oxidative burst response of human peripheral blood neutrophils and monocytes (13). The monocytes were incubated with various concentrations of licochalcone A for 1 h at 37°C, and the cell response to opsonized zymosan was determined. Cell viability after overnight incubation with licochalcone A was determined by trypan blue dye exclusion.

**Monoamine phagocytosis of Candida albicans.** The effect of licochalcone A on human monocyte phagocytosis of *C. albicans* was assessed by a method previously described (17). Briefly, 1 ml of $2 \times 10^8$ human peripheral blood mononuclear clear cells per ml was incubated with 1 ml of licochalcone A in different concentrations in minimal essential medium or medium alone for 30 min at 37°C. After being washed twice, the cells were incubated with 2 ml of $5 \times 10^5 C. albicans$ cells per ml for 30 min at 37°C. The cells were then washed twice, and one portion of the cells was cytocentrifuged and stained for nonspecific esterase. The number of yeast cells ingested by 200 monocytes was determined by direct microscopy, and the number of yeast cells per monocyte was calculated. Another portion of the cells was used for an ultrastructure study (the same procedure as described above).

**Respiration of the parasite (oxygen consumption and changes in carbon dioxide and pH).** A suspension of 4.95 ml of promastigotes (+2 $\times 10^7$/ml) was incubated at 26°C in the presence of 50 g of licochalcone A at different concentrations or medium alone in sealed bottles. Oxygen consumption and changes in carbon dioxide and pH were measured at 2, 4, 8, 24, 48, and 72 h after incubation with an ABL4 acid-base laboratory (Radiometer, Copenhagen, Denmark).

**Activity of mitochondrial dehydrogenases.** The effect of licochalcone A on the activity of parasite mitochondrial dehydrogenases was determined by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium method as described previously (2). This method was developed from the (3,4-dimethylthiazol-2yl)-5-2(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium, which is converted into a soluble, formazan-like dye complex by the parasite mitochondrial dehydrogenase. Briefly, 5 $\times 10^6$ promastigotes were seeded in 96-well flat-bottom microtits and incubated with different concentrations of licochalcone A or medium alone at 26°C. After 3, 6, 12, 24, 48, and 72 h, 25 g of 3-(4,5-dimethylthiazol-2yl)-5-2(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium bromide–phenazine methosulfate was added to each well and further incubated for 3 h at 37°C, whereby the optical densities 492 nm were measured directly with a Titre-Tech 96-well scanner.

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

**RESULTS**

Electron microscopic studies were carried out to determine possible ultrastructural changes in parasites incubated with licochalcone A. Unaffected control promastigotes contained long, slender, dark mitochondria (Fig. 1A). After addition of only 1 $\mu$g of licochalcone A per ml, some ultrastructural changes were induced in the mitochondria because of enlargement and disruption of their structure (Fig. 1B). Increasing concentrations of licochalcone A of up to 5 $\mu$g/ml aggravated these changes, but some cristae were still found intact (Fig. 1C and D). After addition of 10 $\mu$g of licochalcone A per ml, the deformed and swollen mitochondria filled up a great part of the cytoplasm of the promastigotes (Fig. 1E and F). In all of these experiments, the other organelles appeared to be unaffected and no morphological changes were seen in the kinetoplast (Fig. 1F). The kinetoplast is a section of the mitochondrion located just below the basal body of the flagellum.

Phagocytozed amastigotes showed a normal ultrastructure in human MDM grown in normal medium (Fig. 2A and B). Increased concentrations of licochalcone A changed the ultrastructure of the engulfed amastigotes, and 5 $\mu$g of licochalcone A per ml produced an enlarged mitochondrion (Fig. 2C and D) with no damaging effect on the mitochondria of the macrophage (Fig. 2D). When the concentration of licochalcone A was increased to 10 $\mu$g/ml, very marked morphological changes were induced in the amastigotes (Fig. 2E), and again no ultrastructural alteration was observed in the mitochondria of human MDM (Fig. 2E).

Human monocytes grown in medium with 10 $\mu$g of licochalcone A per ml showed no ultrastructural defect in the mitochondria or other cell organelles (Fig. 3A and B). The normal phagocytic effect of human monocytes on *C. albicans* induced no change in the ultrastructure of the mitochondria of the monocytes (Fig. 3C and D), and after addition of 10 $\mu$g of licochalcone A to the medium, no damaging effect on the mitochondria (Fig. 3E and F) or other cell organelles was seen. The average number of *C. albicans* yeast cells per monocyte incubated in the presence of 10 $\mu$g of licochalcone A per ml was 1.9, compared with 2.1 in the control monocytes, indicating that licochalcone A did not influence the phagocytosis of *C. albicans* by human monocytes.

Table 1 shows that in human monocytes about 45 and 40% of the mitochondria of monocytes were influenced by licochalcone A at concentrations of 80 and 160 $\mu$g/ml, respectively, and all of the mitochondria of monocytes were affected at a concentration of 320 $\mu$g/ml. At a concentration of 500 $\mu$g of licochalcone A per ml, the chemiluminescence response of human monocytes was inhibited by 70%, whereas 90% of the cells were viable.

Figure 4 shows that licochalcone A markedly inhibited the oxygen consumption of the parasites, the accumulation of CO$_2$, and the pH decline in the parasite culture. After 2 h of incubation, the concentration of oxygen in control promastigotes was 22.6 ± 0.66 kPa, and after 72 h it dropped sharply to 12.8 ± 0.79 kPa. After 72 h of incubation of the parasites with 5 or 10 $\mu$g of licochalcone A per ml, there were no or very slight changes in the oxygen contents (Fig. 4A). The CO$_2$ content of the parasite cultures was slightly increased in the presence of 5 $\mu$g of licochalcone A per ml and unchanged in the presence of 10 $\mu$g/ml (Fig. 4B). Similarly, there were no changes in the pH of the culture of parasites in the presence of 5 or 10 $\mu$g of licochalcone A per ml (Fig. 4C).

A time- and dose-dependent inhibitory effect of licochalcone A on the activity of parasite mitochondrial dehydrogenase was found (Fig. 5). When promastigotes were incubated with licochalcone A for a longer period, the inhibitory effect on the activity of parasite mitochondrial dehydrogenase was stronger and the dose of licochalcone A required was much less. The 50% inhibitory concentration of licochalcone A after 48 h of incubation was 1.7 $\mu$g/ml. The parasites died after 48 to 72 h of incubation. At higher concentrations (>20 $\mu$g/ml), the parasites died more rapidly. This indicates that the inhibition of mitochondrial activity occurs earlier and at concentrations lower than parasiticidal concentrations.

**DISCUSSION**

Previously we have demonstrated that licochalcone A exhibits strong antileishmanial and antimalarial activities (5–7). However, the mechanism by which licochalcone A kills the parasites and protects animals from infection is not known. It is important to understand the mechanism of action of the antiprotozoal activity of licochalcone A. Our preliminary studies showed that licochalcone A destroyed the mitochondria of *Leishmania* promastigotes (5). In this study, we further examined the effects of licochalcone A on the ultrastructure and function of the mitochondria of *L. major* parasites. The data indicate that licochalcone A alters the ultrastructure and function of the mitochondria of *Leishmania* parasites.

Electron microscopic studies showed that the ultrastructure of the mitochondria of promastigotes and amastigotes was changed in a concentration-dependent manner. In promastigotes, the mitochondria showed moderate edema with a low concentration of licochalcone A and greatly swollen organelles developed with 10 $\mu$g of licochalcone A. The same course of
FIG. 1. Representative electron micrographs of promastigotes incubated in medium alone (A) or in the presence of 1 (B), 5 (C and D), or 10 (E and F) μg of licochalcone A per ml. (A) Sections of two promastigotes showing dense, slender mitochondria (arrowheads). Magnification, ×13,400. Bar, 1 μm. (B) Section of a promastigote with slightly enlarged mitochondria (arrowheads). Magnification, ×20,100. Bar, 0.5 μm. (C) A promastigote in which only the mitochondria seem to be enlarged (arrowheads). Magnification, ×17,600. Bar, 0.5 μm. (D) Higher magnification of one of the affected mitochondria with preserved cristae (arrows). Magnification, ×107,300. Bar, 0.1 μm. (E) Promastigotes with enlarged and disrupted mitochondria (arrowheads). Magnification, ×13,400. Bar, 1 μm. (F) Higher magnification showing part of an unaffected kinetoplast (k) and a mitochondrial crista (arrow). Magnification, ×107,300. Bar, 0.1 μm.
FIG. 2. Representative electron micrographs of human MDM infected with amastigotes and incubated in medium alone (A and B) or in the presence of 5 (C and D) or 10 (E) µg of licochalcone A per ml. (A) Part of an MDM infected with two normal-looking amastigotes (arrowheads). Magnification, ×13,400. Bar, 1 µm. (B) Higher magnification of part of one of the amastigotes showing a normal-looking mitochondrion with cristae (arrows). Magnification, ×54,700. Bar, 0.2 µm. (C) Part of an MDM with an affected amastigote (arrowhead). Magnification, ×9,700. Bar, 1 µm. (D) At a higher magnification, the affected amastigote shows an enlarged and disrupted mitochondrion with some intact cristae (arrows). Outside a normal-looking mitochondrion (m) from the macrophage is visible. Magnification, ×44,300. Bar, 0.2 µm. (E) MDM with two strongly degenerated amastigotes (arrowheads). Note the normal structure of the macrophage mitochondria (m). Magnification, ×13,400. Bar, 1 µm.
FIG. 3. Representative electron micrographs of human monocytes incubated with 10 μg of licochalcone A per ml (A and B), infected with C. albicans without licochalcone A (C and D), and infected with C. albicans after incubation with 10 μg of licochalcone A per ml (E and F). (A) Part of a monocyte with a high number of normal-looking mitochondria (m). Magnification, ×17,600. Bar, 0.5 μm. (B) Higher magnification of one of the mitochondria with normal-looking cristae (arrows) and matrix. Magnification, ×107,400. Bar, 0.1 μm. (C) Monocytes with phagocytized C. albicans (ca). Magnification, ×10,000. Bar, 1 μm. (D) High magnification of the mitochondrion indicated by the arrow in panel C, showing normal ultrastructure. Magnification, ×107,400. Bar, 0.1 μm. (E) MDM with phagocytized C. albicans (ca). Magnification, ×10,000. Bar, 1 μm. (F) High magnification of two mitochondria indicated by the arrow in panel E, showing normal ultrastructure. Magnification, ×107,400. Bar, 0.1 μm.
events was seen in amastigotes, except that in amastigotes other cell organelles were also heavily damaged. Cell organelles of macrophages, however, disclosed no ultrastructural changes at concentrations below 80 \( \mu \text{g/ml} \). Thus, it seems that the effect of licochalcone A on amastigotes is followed by killing of the parasites by macrophages. Furthermore, the oxidative burst response, as measured by the chemiluminescence and phagocytic function of macrophages, was not influenced by fairly high concentrations of licochalcone A. As we have shown previously, oral administration of licochalone A to rats at a dose of 1,000 \( \text{mg/kg} \) of body weight once a day for 2 weeks caused no observable toxicity (6).

Several methods were employed to examine the effect of licochalcone A on the function of parasite mitochondria. It was shown that licochalcone A inhibited the respiration of the parasites in a concentration-dependent manner, as shown by inhibition of \( \text{O}_2 \) consumption and \( \text{CO}_2 \) production by the parasites (Fig. 4). Licochalcone A also exhibited a dose-dependent inhibitory effect on the activity of the mitochondrial dehydrogenase of \( L. \) major promastigotes. The mitochondrial dehydrogenase activity measured in this assay includes the activities of various dehydrogenases in the mitochondrion, such as lactate and malate dehydrogenases (12) and succinate dehydrogenase (14), and has been shown to be mitochondrial specific (10, 16).

The inhibition of the mitochondrial function of the parasite correlated well with changes in the ultrastructure of the mitochondria shown by electron microscopy. These findings indicate that licochalcone A targets the parasite respiratory chain and mitochondria directly.

It was reported that some well-known antiprotozoal drugs and some potential antiprotozoal compounds influence the energy metabolism of the parasite. For example, sodium stibogluconate inhibits phosphorylation of ADP to ATP and the citric acid cycle of \( L. \) mexicana amastigotes (3, 4), and pentamidine isethionate damages the mitochondria of both promastigotes and amastigotes and the kinetoplast DNA core (9). Mycotoxin MT81, an antibacterial, antifungal, and antileishmanial compound isolated from \( \text{Penicillium nigricans} \), inhibits the respiration of \( L. \) donovani promastigotes (15). Atovaquone, a new antimalarial drug developed by Wellcome, inhibits \( \text{Plasmodium falciparum} \) dihydroorotase dehydrogenase, which is the major component of the electron transport chain of intraerythrocytic malaria parasites (11). Studies on several enzymes of the electron transport chain in mitochondria are

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<th>Licochalcone A concn ( (\mu \text{g/ml}) )</th>
<th>% Mitochondria with normal morphology</th>
<th>Chemiluminescence (% of control)</th>
<th>Viability (%)</th>
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\(^a\) The chemiluminescence and viability experiments were repeated three times, and data from one representative experiment are given. Mitochondrial ultrastructure of monocytes incubated in the presence of various concentrations of licochalcone A was examined under an electron microscope. Mitochondria with normal morphology or dilated were counted in 15 cells.

\(^b\) ND, not done.
warranted to document further the exact mechanism of action of licochalcone A on parasite mitochondria. Elucidation of the mechanism of action of this class of compounds is important for the development of these compounds into an antileishmanial and antimalarial drug.

In conclusion, the main discovery presented in this report is that licochalcone A alters the ultrastructure and function of the mitochondria of *Leishmania* parasites but not the mitochondria of host cells.

ACKNOWLEDGMENTS

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REFERENCES


