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*Report*

# *In Vivo* Efficacy of Antifungal Oxoaporphine Alkaloids in Experimental Disseminated Candidiasis

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The efficacy of three antifungal oxoaporphine alkaloids, liriodenine, liriodenine methiodide, and oxoglaucine methiodide, was determined in a mouse model of disseminated candidiasis. Mice infected with a lethal dose of *Candida albicans* NIH B311 were administered varying doses of each drug intraperitoneally or intravenously 7 hr postinfection. Reductions in the number of colony-forming units (CFU) recovered per milligram of kidney tissue were observed in drug-treated animals compared to vehicle-treated control mice. Significance was determined by the Wilcoxon nonparametric rank sum test. Intravenous administration of both liriodenine and liriodenine methiodide resulted in a significant reduction in the number of recovered CFU, while there was no significant response to treatment with oxoglaucine methiodide.

KEY WORDS: candidiasis; antifungal; oxoaporphine; liriodenine; liriodenine methiodide; oxoglaucine methiodide.

## INTRODUCTION

The need for new, more effective, and less toxic antifungal antibiotics for the treatment of disseminated mycotic infections is obvious in light of the significant toxicities and failure rates of the currently available systemic antifungal agents. This problem has become particularly relevant in view of the fact that opportunistic disseminated mycoses is a common complication of acquired immune deficiency syndrome (AIDS). The discovery of new antibiotics has in the past successfully relied primarily upon the isolation of such agents from natural sources. The major advantage of this approach over chemical synthesis or modification of existing agents is the probability of identifying new prototype drugs with quite different chemical structures and, hence, dissimilar toxicities and cross-resistance with present drug therapies. Although microorganisms have traditionally served as the primary source for new antibiotics, it has recently been shown that higher plants also serve as sources for a number of diverse antimicrobial agents (1–5). One such example is the antifungal oxoaporphine alkaloid, liriodenine (I) (Fig. 1) a constituent of the heartwood of *Liriodendron tulipifera*, commonly known as the tulip tree (3). Using a bioassay-directed fractionation procedure, liriodenine (I) was isolated and identified as the major antimicrobial constituent of *L. tulipifera*. Conversion of liriodenine to its methiodide salt (II) significantly increased its *in vitro* antimicrobial activity (3) and has sparked further interest in the assessment of the therapeutic potential of these compounds. Further studies with these two compounds and a related semisynthetic alkaloid, oxoglaucine methiodide (III), confirmed the *in vitro* anticandidal activity of all three alkaloids. The results of the evaluation of the *in vivo* efficacy of these alkaloids are described in this report.

## MATERIALS AND METHODS

### General

*Candida albicans* strain NIH B311 (6) was used to induce experimental disseminated candidiasis in mice. Cultures of the organism were either lyophilized or stored under sterile mineral oil and subcultures on Sabouraud dextrose agar (SDA)[4] were prepared as needed. Subcultures were incubated at 37°C. For short-term maintenance, cultures on SDA were stored at 4°C.

Liriodenine (I) was isolated from the heartwood of *Liriodendron tulipifera* L. as previously described (3). Liriodenine methiodide (II) (3) was prepared by treatment of a chloroform solution of liriodenine with methyl iodide at reflux. Oxoglaucine methiodide (III) was prepared by treatment of a refluxing benzene solution of oxoglaucine with iodomethane as previously described (3). Amphotericin B was purchased from Sigma Chemical Co.

Female ICR mice (Charles River Breeding Laboratories) weighing 20–25 g each were housed in microisolator

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[4] Abbreviations used: CFU, colony-forming units; PSS, physiological sterile saline; SDA, Sabouraud dextrose agar; SDB, Sabouraud dextrose broth; MIC, minimum inhibitory concentration; AMB, amphotericin B; ATCC, American Type Culture Collection



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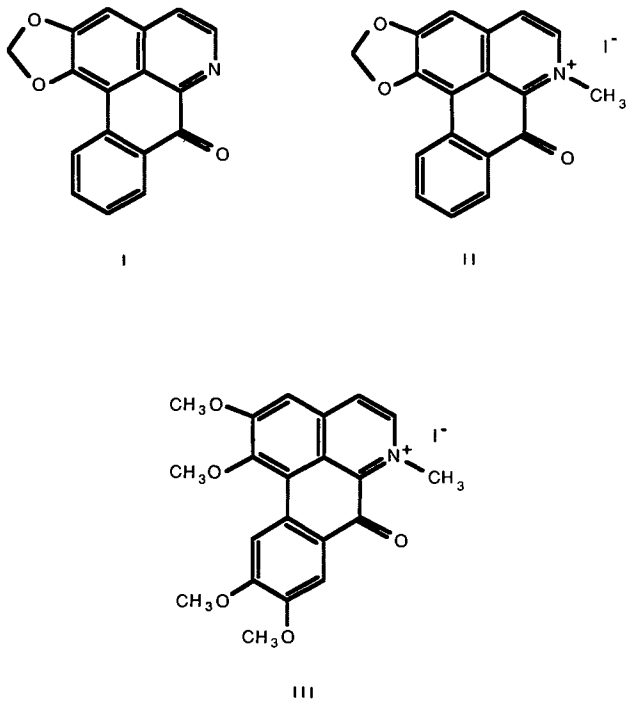

Fig. 1. Structures of liriodenine (I), liriodenine methiodide (II), and oxoglaucine methiodide (III).

cages (Lab Products, Maywood, N.J.) equipped with HEPA filters and were administered food and water *ad libitum*. Animals were maintained in air-conditioned rooms at 72–74°F on a 12-hr light, 12-hr dark cycle. Animals were quarantined and acclimated 1 week prior to the initiation of experiments.

### Evaluation of Efficacy in Disseminated Candidiasis

A mouse model of disseminated candidiasis previously described by Rabinovich *et al.* (7), with some modifications, was utilized for the evaluation of *in vivo* efficacy. Acute, disseminated infections in mice were produced by intravenous injection (via tail vein) of $10^6$ colony-forming units (CFU) of *C. albicans* NIH B311 in sterile physiological saline solution (PSS). Cell suspensions for injection were prepared by incubation of *C. albicans* B311 in Sabouraud dextrose broth (SBD) at 37°C for 4–6 hr, at which time the cells were sonicated briefly, centrifuged, washed once, and suspended in PSS to the appropriate dilution, based on direct count by hemacytometer. The reliability of the hemacytometer count was verified by viability determination (by triplicate plating of aliquots of the PSS suspension) which showed greater than 96% of the cells as viable CFU. The dose of $10^6$ CFU/mouse was verified as lethal within 7–10 days and capable of induction of systemic candidiasis in mice, as evidenced by proliferation of *C. albicans* in the kidneys, within 2 hr postinoculation. Preconditioning of the mice to suppress the immune system was not necessary when log-phase (4–6 hr) cultures were used as the inoculum.

Seven hours following intravenous inoculation with *C. albicans*, groups of mice received varying doses of liriodenine methiodide (II), liriodenine (I), or oxoglaucine methiodide (III) intraperitoneally (i.p.) or intravenously (i.v.). Animals were sacrificed at 24 hr postinfection and the kidneys were aseptically removed, weighed, and homogenized in 5 ml of PSS. Following 10-fold serial dilutions from the homogenized kidney suspension (using sterile PSS as diluent), a volume of 0.01 ml was cultured in triplicate on SDA plates which were incubated for approximately 16 hr. The mean numbers of CFU of *C. albicans* per milligram of kidney tissue were determined for each group and the Kruskal–Wallis test was applied to the entire experimental group. Each individual treatment group was compared to the control group by the Wilcoxon nonparametric rank sum test using $P < 0.05$ as a test of significance.

### Estimation of the Median Lethal Dose

The method of Litchfield and Wilcoxon (8) was used to estimate the median lethal dose ($LD_{50}$) of liriodenine, liriodenine methiodide, and oxoglaucine methiodide. Groups of 10 mice (equal male and female) were administered the test compound and observed for morbidity and lethality for 14 days. Doses were doubled or halved until the maximum dose was found which produced no lethality and a minimal dose was found which produced 100% lethality within a 14-day period. Efforts were made in every case to determine three doses within the all kill–no kill range and an estimated $LD_{50}$ was obtained by probit analysis. When the supply of sample precluded numerous dose determinations, the $LD_{50}$ is reported as a range.

### Determination of Minimum Inhibitory Concentration (MIC) Values

The twofold serial broth dilution assay (3) in Mycophil broth (BBL Microbiology Systems) was used to determine the MIC values. In addition to *C. albicans* B311, the MIC values for liriodenine, liriodenine methiodide, and oxoglaucine methiodide were also determined for two additional strains of *C. albicans:* ATCC 10231 and a clinical isolate designated WH. All compounds were initially tested using a concentration of 100 μg/ml in the first tube. The test compound was added to sterile SDB as a solution. The test organisms were grown in SDB for 24 hr at 37°C, at which time the cells were harvested by centrifugation (2000 rpm). After centrifugation, the cells were washed and suspended in sterile PSS to give a final concentration of $10^6$ CFU/ml (adjusted using a hemacytometer). Using a calibrated sterile inoculating loop, 10 μl of the $10^6$ CFU/ml suspension of *C. albicans* was used as the inoculum for each tube. The MIC value was taken as the lowest concentration of compound that inhibited the growth of the test organisms after 24 and 48 hr of incubation at 37°C. The antifungal agent amphotericin B (AMB) was included as standard in each screen. Subcultures onto SDA were made from the tube containing the MIC.

## RESULTS AND DISCUSSION

Based on the MIC values, the most active of the three related antifungal oxoaporphine alkaloids is liriodenine methiodide (II), with an MIC value of 0.78 μg/ml, while liriodenine (I) and oxoglaucine methiodide (III) are approximately equal in activity, with MIC values of 3.12 μg/ml for each.

Prior to the initiation of *in vivo* efficacy studies, a deter-

mination of the acute toxicity, as the median lethal dose ($LD_{50}$), of each compound was undertaken. The $LD_{50}$ of liriodenine methiodide is 14.3 mg/kg following i.v. administration and between 50 and 100 mg/kg following i.p. administration.[5] The reported $LD_{50}$ for amphotericin B following i.v. administration is between 1 and 4 mg/kg (9,10). Liriodenine appears to be less toxic (as $LD_{50}$) than liriodenine methiodide, with $LD_{50}$ values of 120 mg/kg following i.v. administration and >250 mg/kg following i.p. administration. Oxoglaucine methiodide (3) has an $LD_{50}$ of 12.2 mg/kg following i.v. administration and between 75 and 100 mg/kg following i.p. administration.

The *in vivo* efficacy of each compound was determined in mice infected with a lethal dose of *C. albicans* NIH B311 (via i.v. injection). Mice treated with liriodenine methiodide and liriodenine were found to exhibit consistently a significant reduction in the number of recovered CFU as compared to vehicle-treated control groups.

Following single-dose i.p. administration of liriodenine methiodide (II) 7 hr postinoculation, a significant reduction ($P < 0.05$) in the number of CFU recovered from the kidneys (following sacrifice at 24 hr postinfection) was observed with all dosages as compared to vehicle-treated control mice (Table I). As indicated in Table I, the significance level of the 0.1-mg/kg dose was not within 95% confidence intervals; however, this dose was between two doses which did show a significant reduction ($P < 0.05$). Since this seemed inconsistent, two additional studies were performed in which liriodenine methiodide was administered at a dose of 0.1 mg/kg body weight. In both studies, the number of recovered CFU was significantly reduced in liriodenine methiodide-treated animals as compared to vehicle-treated control animals ($P = 0.004$, 90% reduction, and $P = 0.026$, 97% reduction). In early studies, AMB was used as a positive control at a dose of 0.1 mg/kg, at which no significant reduction in the recovered CFU was observed as compared to vehicle-treated control mice. In subsequent studies, it was found that a dose of 0.5 mg AMB/kg consistently led to a significant reduction (>90%) in recovered CFU in treated mice vs vehicle-treated infected mice.

There was also significant reduction in the number of recovered CFU per milligram of kidney tissue following a single i.v. dose of liriodenine methiodide (Table I). It is interesting to note that the optimum effects of liriodenine methiodide were observed at doses of 0.5 and 0.1 mg/kg. At higher doses of 2.0 and 1.5 mg/kg there appears to be less efficacy, as evidenced by a $P > 0.05$ and a lower percentage reduction in the number of recovered CFU. These results were verified by duplicate evaluation and were also observed with liriodenine.

Following multiple-dose i.p. administration of liriodenine methiodide, remarkably good efficacy was observed, as determined by a reduction in recovered CFU (Table II). It therefore appears that a multiple-dose regimen is more efficaceous than single-dose treatment.

Liriodenine methiodide (II) appears to be effective by either i.v. or i.p. single-dose administration, particularly at doses of 0.1 and 1.0 mg/kg. However, a multiple-dose regimen by the i.p. route appears to be the most effective, particularly at a dose of 0.5 mg/kg. At this dose, liriodenine methiodide is not significantly different from AMB in its ability to reduce the number of CFU recovered from kidney.

[5] Due to limitation in the sample supply, complete $LD_{50}$ values were obtained for each alkaloid following i.v. administration only, with ranges determined for i.p. administration.

**Table I.** Counts (CFU/g) of *Candida albicans* in Kidneys Following Single-Dose Treatment with Liriodenine Methiodide[a]

| Dose, mg/kg (*N*) | Route of administration | CFU/g × $10^6$ (range) | *P* | % reduction |
|---|---|---|---|---|
| Control (9) | i.p. | 8.13 (0.46–30.6) | — | |
| 1.0 (5) | i.p. | 1.20 (0.58–1.91) | 0.041 | 85.2 |
| 0.1 (5) | i.p. | 1.53 (0.54–2.5) | 0.095 | 81.2 |
| 0.05 (5) | i.p. | 1.26 (0.03–3.91) | 0.021 | 84.5 |
| 0.01 (5) | i.p. | 3.29 (1.55–5.48) | NS | 59.5 |
| AMB, 0.1 (3) | i.p. | 3.28 (2.24–3.81) | NS | 59.7 |
| Control (5) | i.v. | 28.8 (4.33–60.1) | — | |
| 2.0 (6) | i.v. | 22.7 (1.52–36.4) | NS | 21.2 |
| 1.5 (5) | i.v. | 21.9 (2.22–45.9) | NS | 24.0 |
| 1.0 (5) | i.v. | 4.05 (2.89–5.99) | 0.032 | 85.9 |
| 0.5 (4) | i.v. | 3.52 (1.71–5.54) | 0.032 | 87.8 |
| 0.1 (5) | i.v. | 13.7 (3.36–22.3) | NS | 52.4 |
| AMB, 0.5 (4) | i.v. | 2.10 (0.08–5.56) | 0.016 | 92.7 |

[a] Mice were injected i.v with $10^6$ CFU of *Candida albicans* B311. Liriodenine was administered 7 hr postinfection.

**Table II.** Counts (CFU/g) of *Candida albicans* in Kidneys Following Multiple-Dose Intraperitoneal Treatment with Liriodenine Methiodide[a]

| Dose, mg/kg (*N*) | CFU/g × $10^6$ (range) | *P* | % reduction |
|---|---|---|---|
| Control (4) | 278 (1.3–1210) | — | |
| 1.0 (6) | 1.52 (0.79–3.48) | 0.026 | 99.5 |
| 0.5 (6) | 0.62 (0.05–1.0) | 0.002 | 99.8 |
| 0.1 (5) | 1.29 (0.53–1.99) | 0.048 | 99.5 |
| 0.01 (6) | 365 (0.5–929) | NS | 0.00 |
| AMB, 0.5 (5) | 0.04 (0.01–0.07) | 0.008 | 99.9 |

[a] Mice were injected i.v. with $10^6$ CFU of *Candida albicans* B311. Liriodenine methiodide was administered 2, 24, and 42 hr postinfection.

While the parent compound liriodenine (I) was less active *in vitro* than its methiodide salt, it was nevertheless considered a promising candidate for *in vivo* evaluation in light of its very low acute toxicity ($LD_{50}$) and relatively good MIC value. The *in vivo* efficacy of liriodenine was determined in the same manner as for liriodenine methiodide. Following single-dose i.p. administration (7 hr postinfection), liriodenine also exhibited a relatively good efficacy at 1.0 and 0.5 mg/kg body weight (Table III).

Following single-dose i.v. administration of liriodenine, a significant reduction in the number of recovered CFU was observed at doses of 0.5 and 1.0 mg/kg (Table III). As was the case with liriodenine methiodide, higher doses of liriodenine appear to be *less* efficaceous than lower doses. Duplication of these studies verified this phenomenon, i.e., liriodenine was effective over a narrow dose range (0.1–1.0 mg/kg). While the cause of this phenomenon is unknown, one could speculate that at the higher doses liriodenine and liriodenine methiodide may exhibit some subacute toxicity which could be manifested as an apparent loss of antifungal efficacy. Clearly, extensive further studies would be required to establish the cause of this observation.

**Table III.** Counts (CFU/g) of *Candida albicans* in Kidney Following Single-Dose Treatment with Liriodenine[a]

| Dose, mg/kg (*N*) | Route of administration | CFU/g × $10^7$ (range) | *P* | % reduction |
|---|---|---|---|---|
| Control (6) | i.p. | 5.62 (0.04–13.4) | — | — |
| 1.0 (6) | i.p. | 0.77 (0.04–1.48) | 0.032 | 86.3 |
| 0.5 (6) | i.p. | 0.32 (0.04–1.00) | 0.021 | 94.3 |
| 0.1 (7) | i.p. | 1.36 (0.09–3.51) | NS | 75.8 |
| AMB, 0.5 (7) | i.p. | 0.36 (0.003–1.74) | 0.049 | 99.4 |
| Control (5) | i.v. | 14.7 (1.4–32.7) | — | — |
| 5.0 (4) | i.v. | 7.03 (5.9–8.36) | NS | 52.2 |
| 1.0 (9) | i.v. | 9.30 (7.53–11.5) | NS | 36.7 |
| 0.5 (5) | i.v. | 0.84 (0.11–1.59) | 0.016 | 94.3 |
| 0.1 (5) | i.v. | 3.56 (1.51–9.44) | 0.075 | 75.8 |
| AMB, 0.5 (4) | i.v. | 0.019 (0.005–0.032) | 0.008 | 99.9 |
| Control (5) | p.o. | 11.6 (0.77–23.7) | — | — |
| 30.0 (6) | p.o. | 2.58 (0.28–5.24) | 0.041 | 77.8 |
| 20.0 (5) | p.o. | 4.81 (1.82–8.35) | 0.041 | 58.5 |
| 12.5 (6) | p.o. | 4.17 (0.71–10.5) | 0.063 | 64.1 |
| KTZ, 5.0 (6) | p.o. | 4.22 (2.23–5.91) | 0.063 | 63.6 |

[a] Mice were injected i.v. with $10^6$ CFU of *Candida albicans* B311. Liriodenine was administered 7 hr postinfection.

The efficacy of liriodenine was also evaluated following oral administration, using ketoconazole as a positive control. While a range of doses was evaluated (50, 30, 20, 12.5, 6.25, and 3.12 mg/kg), the only doses which afforded a verified reduction in recovered CFU were 30, 20, and 12.5 mg/kg (Table III).

While oxoglaucine methiodide appeared to be as active as liriodenine *in vitro*, it did not appear to be as effective *in vivo*. Following i.p. administration (7 hr postinfection), oxoglaucine methiodide did not exhibit a significant reduction at any of the dosage levels evaluated (10.0, 5.0, 2.0, 1.5, 1.0, 0.5, and 0.1 mg/kg).

Based on the data observed from each of these experiments, it can be concluded that liriodenine methiodide (II) is therapeutically effective against systemic *C. albicans* infection in mice. Maximum activity of liriodenine methiodide against *C. albicans* infection was observed at doses between 0.1 and 1.0 mg/kg body weight regardless of the treatment regimen. However, the efficacy of liriodenine methiodide at these doses appears to be greatly enhanced if the drug is administered at 2, 24, and 42 hr postinfection.

Liriodenine (I) also exhibits some level of efficacy, with the added advantage of being orally effective. Additional studies to determine the efficacy of these drugs at additional dosage levels and, particularly, at varying dosage intervals are currently in progress.

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