Inhibition of Growth of the Dimorphic Fungus
Paracoccidioides brasiliensis by Ajoene

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Ajoene, a garlic-derived compound that prevents platelet activation, inhibited the growth of Paracoccidioides brasiliensis, a fungal pathogen for humans, by affecting the integrity of the fungal cytoplasmic membrane. This action may be the basis for the study of ajoene as a possible specific antifungal drug.

Garlic (Allium sativum) has been used for centuries as a folk medicine (5). One of the reported uses concerns its putative antymycotic and antibacterial action (5), which has been traditionally attributed to the presence of allicin (Fig. 1) (10, 14). A novel compound derived from garlic, ajoene (Fig. 1), which is a potent inhibitor of platelet aggregation (1–3), also possesses in vitro antifungal activity against Aspergillus niger and Candida albicans (15). We explored its effect on the in vitro growth of Paracoccidioides brasiliensis, a pathogenic dimorphic fungus which causes one of the commonest systemic mycoses in Latin American peasants (12).

P. brasiliensis IVIC Pb73 has been maintained on Sabouraud liquid broth (BBL Microbiology Systems)-modified agar slants. Yeast (Y) and mycelial (M) phases were grown in PYG medium (peptone [1.5 g], yeast extract [1.5 g], and glucose [0.5 g] in 100 ml of distilled water [final pH, 7.0]), with continuous shaking (11) for up to 5 days at 37°C (Y phase) and at 23°C (M phase). Synthetic ajoene (M₉, 234), obtained as previously described (3; R. Apitz-Castro and M. K. Jain, U.S. patent 4665088, May 1987) with minor modifications, was prepared for use as a 100 mM solution in methanol and added to the medium at final ajoene concentrations of 0, 50, 100, and 200 μM.

![Chemical structure of ajoene and its precursors](image)

FIG. 1. Chemical structure of ajoene and its precursors.

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Samples (10 ml) of either Y or M cultures were taken daily from 0 to 5 days and filtered through membrane filters (0.8-μm pore size; Millipore Corp., Bedford, Mass.). Dry weight was determined in each case. Curves were repeated four times in duplicate. Culture media without ajoene and with methanol (100 μl) were used as controls.

For electron microscopic observations, cells grown in 100 ml of medium, with or without ajoene as described above, were collected by membrane filtration after 3 days and treated for inclusion (4) or freeze-etching (6). Samples were...
FIG. 4. Electron microscopy of *P. brasiliensis* Y phase. Thin sections: (a) control, (b) 50 μM ajoene, (c) 100 μM ajoene, (d) 200 μM ajoene (bar, 200 nm). Observe the thickening and disturbance of the cell membrane with increasing amounts of ajoene. Freeze-etching: (e) control, (f) 100 μM ajoene (bar, 600 nm), (g) detail of f (bar, 300 nm). An irregular fracture plane is present in the presence of ajoene. Abbreviations: CW, cell wall; PM, cytoplasmic membrane; PS, periplasmic space; OB, osmiophilic body; E, E face of the membrane.
examined under an electron microscope (JEOL model JEM 100B; Japan Electric and Optic Laboratories, Tokyo).

Ajoene at 50 mM inhibited the normal growth of *P. brasiliensis* by 90% in the Y phase and by 60% in the M form (Fig. 2 and 3). By monitoring this effect under the electron microscope, it could be seen that with 50 μM ajoene (Fig. 4b) the membrane, which was continuous, uniform, and about 12 nm thick in the control cells of the Y phase (Fig. 4a), started to wrinkle and eventually became loosely arranged and detached from the cell wall in some places. With 100 μM ajoene, this effect increased, and the membrane thickened to an average of 60 nm (Fig. 4c). Cell lysis was observed with 200 μM ajoene (Fig. 4d). Freeze-etching suggested that with 100 μM ajoene irregular fracture planes and protuberances were produced in face E of the cytoplasmic membrane (Fig. 4e, f, and g).

Ajoene also inhibited growth in the M phase. The M phase was less affected in its structure by ajoene. At no less than 100 μM ajoene, a moderate thickening of the membrane (20 nm) was observed, accompanied by some discontinuity and
detachment of the membrane from the cell wall (Fig. 5a to d). However, these changes were enough to provoke hyphal deterioration (Fig. 5) and inhibition of growth (Fig. 3). No changes in the fracture planes of the membrane were seen (Fig. 5e and f). Incubation with ajoene induced the formation of electron-dense inclusions in the cytoplasm of both phases. Ajoene did not alter the appearance of the cell wall in any case.

Our present results clearly indicate that ajoene is a potent inhibitor of growth in vitro when tested against the Y and M phases of the pathogenic fungus *P. brasiliensis* (90% inhibition at a concentration of 50 μM or 11.7 μg/ml), comparing favorably with the in vitro susceptibility of this fungus to sulfonamides (8) and to ketoconazole and amphotericin B (9).

From the ultrastructural data, it seems that ajoene causes important changes in the cell membrane, without any noticeable effect on the cell wall. In this regard, preliminary experiments (San-Blas et al., unpublished results) suggest that ajoene does not affect the activity of the enzyme glucan synthetase, which constitutes a crucial step in the synthesis of cell wall components. Previous observations (15) that ajoene damages the fungal cell wall as the primary target may be now reconsidered as a consequence of perturbations in the membrane leading to cell lysis and deterioration of all fungal structures, rather than to a direct action of ajoene against the cell wall.

Contrary to most polyenic and imidazole antifungal drugs, ajoene seems to be highly selective for the fungal membrane, since at concentrations of 200 μM it does not induce any noticeable change in the ultrastructure of blood platelets from human or other mammalian species (1). Moreover, when ajoene is administered intravenously in dogs, the deactivating effect of ajoene on blood platelets is fully reversible in about 3 h (2).

The mechanism of the effect of ajoene on fungi is not yet clear. With respect to the more pronounced effect in the Y phase than in the M form, an earlier report (13) suggests that both phases of *P. brasiliensis* may vary in membrane structure, since their performances in the presence of several polar and nonpolar detergents differed to an important degree, probably because the overall lipid compositions in both phases are different. A reasonable working hypothesis, derived from our knowledge of the effect of the compound in other cells (7), is that the fungus takes up ajoene actively from the medium and that, perhaps due to the existence of a cell wall, the concentration of ajoene in the plasma membrane attains levels that induce a dramatic alteration of the lipid bilayer, leading to cell lysis. However, at present we cannot exclude a selective effect of ajoene on any major metabolic pathway of fungal cells. In any case, ajoene seems to be capable of selectively affecting fungal cells, without deleterious effects on mammalian cells. The potential clinical use of ajoene as an antifungal drug is further substantiated by its lack of toxicity in mammals (up to 25 mg/kg in mice or dogs; Apitz-Castro, unpublished results).

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**LITERATURE CITED**


