Inhibition of *Paracoccidioides brasiliensis* by ajoene is associated with blockade of phosphatidylcholine biosynthesis

Gioconda San-Blas,¹ Julio A. Urbina,² Edgar Marchán,² Lellys M. Contreras,² Françoise Sorais¹ and Felipe San-Blas¹

Author for correspondence: Gioconda San-Blas. Tel: +58 2 504 1040. Fax: +58 2 504 1382.
e-mail: gsanblas@pasteur.ivic.ve

Instituto Venezolano de Investigaciones Científicas (IVIC), Centres of Microbiology and Cell Biology¹ and Biophysics and Biochemistry,² Apartado 21827, Caracas 1020A, Venezuela

In *Paracoccidioides brasiliensis*, a dimorphic fungus pathogenic for humans, no significant differences were observed in the phospholipid species of both morphological phases. The species observed were phosphatidylcholine (PC, 30–40%), phosphatidylethanolamine (PE, 27–28%), phosphatidylserine (16–19%), phosphatidylinositol (13–17%) and sphingomyelin (3–5%). The main fatty acids found in the yeast (Y) phase were palmitate (56%), linoleate (18%) and oleate (15%), while linoleate predominated (61%) in the mycelial (M) phase, followed by palmitate (27%) and oleate (7%). In the Y phase the main free sterol was ergosta-5,22-dien-3 β -ol (82%) plus some lanosterol (12%) and ergosterol (6%), while in the M phase, the latter predominated (88%), followed by low levels of ergosta-5,22-dien-3 β -ol (12%). Ajoene [(*E,Z*)-4,5,9-trithiadodeca-1,6,11-triene 9-oxide], a platelet aggregation inhibitor derived from garlic, induced alterations in phospholipid and fatty acid proportions such that PC was reduced to about 18% in both phases and PE increased to 38% (Y phase) or 44% (M phase), suggesting inhibition of PC synthesis. Ajoene also reduced saturated fatty acids (16:0 and 18:0) from 67 to 35% in the Y phase, with a corresponding increase in the unsaturated components. This effect was not seen in the M phase.

Keywords: fungal dimorphism, *Paracoccidioides brasiliensis*, ajoene, antifungal drugs, fungal lipids

INTRODUCTION

Paracoccidioides brasiliensis is a thermally dimorphic fungus and the causative agent of a prevalent human systemic mycosis in Latin America where it is geographically constrained (Wanke & Londero, 1994). The pathogenic yeast-like (Y) phase grows at 37 °C, while a mycelial (M) phase develops at 23 °C (Lacaz, 1994). Ajoene [(*E,Z*)-4,5,9-trithiadodeca-1,6,11-triene 9-oxide], a platelet aggregation inhibitor derived from garlic (Apitz-Castro *et al.*, 1983), behaves as an antifungal agent against *P. brasiliensis* (San-Blas *et al.*, 1989), *Aspergillus niger* and *Candida albicans* (Yoshida *et al.*, 1987), *Cladosporium carrionii* and *Fonsecaea pedrosoi* (Sánchez-Mirt *et al.*, 1993). Ajoene disturbs the fungal

plasma membrane (San-Blas *et al.*, 1989; Sánchez-Mirt *et al.*, 1993), an effect that is more prevalent in the Y phase in *P. brasiliensis*. Membrane disturbance leads to the deterioration of fungal structures, among them the cell wall, whose synthesis depends on the correct performance of the enzymic machinery located in the plasma membrane (San-Blas & San-Blas, 1994). Ajoene is also capable of blocking the thermally induced transition from the M to the Y phase (San-Blas *et al.*, 1993b), an effect that together with the already mentioned effect on growth, may be of interest in therapy, since the transition process is the first requirement for the establishment of paracoccidioidomycosis in the host (Lacaz, 1994).

Recently, it was shown that ajoene also acts as an antiproliferative drug against the epimastigote and amastigote forms of *Trypanosoma cruzi*, the causative agent of Chagas disease (Urbina *et al.*, 1993), an effect associated with a specific blockade of the *de novo*

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SFA, saturated fatty acids; UFA, unsaturated fatty acids.

synthesis of phosphatidylcholine, the main phospholipid species of these cells. Here we report the effects of ajoene on the lipid composition of Y and M phase cells of *P. brasiliensis*. Our results suggest a biochemical mechanism of action similar to that proposed for *T. cruzi*.

METHODS

Fungus and growth conditions. *P. brasiliensis* strain IVIC Pb73 (ATCC 32071) has been maintained in our laboratory on PYG medium (per litre distilled water: peptone, 5 g; yeast extract, 5 g; glucose, 15 g; final pH 7.0) agar slants for several years. It was grown in PYG liquid medium (200 ml medium in 500 ml Erlenmeyer flasks) after inoculation with 10 ml of a seed culture. The cultures were incubated for 3 d at 37 °C (Y phase) or 23 °C (M phase) with continuous shaking on a gyratory shaker operated at 120 r.p.m. (Sorais-Landáez & San-Blas, 1993). Ajoene (25 mM for the Y phase, 50 mM for the M phase) was added to the culture medium at time zero to inhibit growth by 50% (San-Blas *et al.*, 1989). It was prepared for use as a 100 mM solution in ethanol. Cultures with the addition of equivalent volumes of ethanol in the absence of ajoene were used as controls. Ajoene was the kind gift of Dr R. Apitz-Castro (R. Apitz-Castro & M. K. Jain, US patent 4665088, May 1987).

Lipid analysis. Y and M cultures of *P. brasiliensis* were washed three times by centrifugation (Y phase) or filtration (M phase) and total lipids extracted with chloroform/methanol (2:1; v/v). Polar and neutral lipids were separated by silicic acid column chromatography (Larralde *et al.*, 1988). Polar lipids were fractionated by TLC using high performance plates (Merck 5715) with chloroform/methanol/30% aqueous ammonia (17:7:1, by vol.) as eluent (Cuzner & Davison, 1967); the different species were quantified by measuring organic phosphate content (Ames & Dubin, 1960). Fatty acids esterified to the lipid fraction were converted to their methyl esters by incubation in the presence of 2% H₂SO₄ in methanol at 60 °C for 1 h (Urbina *et al.*, 1993). The neutral lipid fraction was analysed by GLC in a Varian 3700 Gas Chromatograph equipped with a flame ionization detector as described previously (Urbina *et al.*, 1993). For structural assignments, this fraction was also analysed in a capillary high resolution column (25 m × 0.20 mm i.d. Hewlett-Packard HP-5 MS column with 5% phenylmethylsiloxane, 0.33 mm film thickness) using a Hewlett-Packard 5890 Series II Gas Chromatograph equipped with a 5971A Mass Sensitive Detector. The lipids were injected in ethyl acetate and the column kept at 50 °C for 1 min, followed by a temperature increase to 270 °C at a rate of 25 °C min⁻¹ and finally to 300 °C at a rate of 1 °C min⁻¹. The carrier gas (He) flow was kept constant at 1 ml min⁻¹. The injector temperature was fixed at 250 °C and that of the detector at 280 °C.

RESULTS AND DISCUSSION

No significant differences were observed in the phospholipid species of either morphological phase of *P. brasiliensis* (Table 1). Phosphatidylcholine (PC) was the main species in both Y and M cells (30.5 and 39.7%, respectively), followed by phosphatidylethanolamine (PE, 27.7 and 28.7%), phosphatidylserine (PS, 15.8 and 19.0%), and phosphatidylinositol (PI, 13.1 and 17.8%). Therefore, their proposed role in *P. brasiliensis* dimorphism (Manocha, 1980) is questioned. These results

contrast with those found in *C. albicans* whose M form contains significantly higher levels of PC, PS and PI, and lower levels of PE, compared to the Y form (Goyal & Khuller, 1994).

The predominant fatty acid in control Y cells was palmitate (16:0; 56.2%), followed by linoleate (18:2; 18.2%) and oleate (18:1; 14.9%) (Table 2). In the M phase, linoleate was by far the most abundant component (61.1%), followed by palmitate (26.8%) and much lower levels of oleate (5%). Stearate (18:0) was a minor component in both phases (5.5–10.7%). These results agree with those of Manocha (1980) on the predominance of palmitate (16:0) in the Y phase and linoleate (18:2) in the M phase. As reported in *C. albicans* (Ghannoum *et al.*, 1986; Mishra *et al.*, 1992; Goyal & Khuller, 1994), the ratio of unsaturated to saturated fatty acids (UFA/SFA) was higher in the M form of *P. brasiliensis* (Table 2). The higher levels of SFA in the Y form are probably required to maintain normal basic permeability properties of the plasma membranes at its relatively high growth temperature (Goyal & Khuller, 1994). As in *P. brasiliensis*, the lowering of growth temperature increases fatty acid unsaturation in *Aspergillus niger*, *Penicillium chrysogenum* and *Trichoderma reesei*, but not in *Neurospora crassa* where unsaturation remains unchanged (Suutari, 1995).

Ajoene is an inhibitor of *in vitro* growth of *P. brasiliensis* (San-Blas *et al.*, 1989), although not as potent as sulfonamides (Restrepo & Arango, 1980), azoles and amphotericin B (Restrepo *et al.*, 1984; San-Blas *et al.*, 1993a). It provokes important changes in the plasma membrane, without any noticeable direct effect on the cell wall (San-Blas *et al.*, 1989). Analysis of the cellular phospholipid content showed that ajoene induced a reversal of the relative proportion of the main phospholipid species in both Y and M phases (Table 1), leading to a predominance of PE (38.2 and 43.6% in the Y and M phases, respectively), in contrast with data from untreated cells. The proportions of PS (14.6 and 20.9%, respectively), PI (13.1 and 18.1%) and sphingomyelin (2.7 and 5.0%) were not significantly affected. Therefore, while ajoene (200 µM) does not induce any marked changes in the ultrastructure of blood platelets from human or other mammalian species (Apitz-Castro *et al.*, 1988), it seems highly selective for the fungal membrane where it modifies the headgroup composition of *P. brasiliensis* phospholipids, similar to the effect of aqueous garlic extracts (AGE) on *C. albicans* (Adetumbi *et al.*, 1986; Ghannoum, 1988). Compositional changes of this type should lead to a very unstable lipid bilayer, consistent with the ultrastructural results obtained previously (San-Blas *et al.*, 1989). Treatment of Y cells with ajoene (Table 2) also produced a drastic reduction in SFA (16:0 and 18:0) (67%–35%) with a concomitant increase (33%–65%) of the UFA (18:1 and 18:2), leading to a UFA/SFA ratio similar to that of the M phase. The large increase in UFA in the Y phase could lead to loss of vital cytoplasmic components in the treated cells. On the other hand, no significant modifica-

Table 1. Effect of ajoene on phospholipid composition of *P. brasiliensis*

The results, expressed as mol%, are means $\pm$ SEM of three independent experiments.

Phospholipid species	Y phase		M phase	
	Control	+ 25 mM ajoene	Control	+ 50 mM ajoene
PC	30.5 $\pm$ 4.1	17.2 $\pm$ 2.5	39.7 $\pm$ 3.8	19.4 $\pm$ 3.6
PE	27.7 $\pm$ 3.4	38.2 $\pm$ 4.0	28.7 $\pm$ 3.5	43.6 $\pm$ 5.0
PS	19.0 $\pm$ 2.4	20.9 $\pm$ 2.5	15.8 $\pm$ 2.8	14.6 $\pm$ 3.0
PI	17.8 $\pm$ 2.3	19.8 $\pm$ 2.6	13.1 $\pm$ 2.6	18.5 $\pm$ 3.0
Sphingomyelin	5.0 $\pm$ 1.2	3.9 $\pm$ 1.1	2.7 $\pm$ 0.9	3.9 $\pm$ 1.0

Table 2. Effect of ajoene on fatty acid composition of *P. brasiliensis*

The results, expressed as mol%, are means $\pm$ SEM of three independent experiments.

Fatty acid species	Y phase		M phase	
	Control	+ 25 mM ajoene	Control	+ 50 mM ajoene
16:0	56.2 $\pm$ 5.3	32.3 $\pm$ 3.6	26.8 $\pm$ 1.1	28.7 $\pm$ 1.5
18:0	10.7 $\pm$ 3.2	2.6 $\pm$ 0.5	5.5 $\pm$ 0.3	6.3 $\pm$ 1.0
18:1	14.9 $\pm$ 0.7	23.9 $\pm$ 3.8	6.6 $\pm$ 0.1	5.0 $\pm$ 1.7
18:2	18.2 $\pm$ 4.0	41.2 $\pm$ 1.9	61.1 $\pm$ 1.3	60.0 $\pm$ 4.2
SFA	66.9 $\pm$ 6.9	34.9 $\pm$ 4.2	32.3 $\pm$ 1.5	35.0 $\pm$ 2.3
UFA	33.1 $\pm$ 4.5	65.1 $\pm$ 5.5	67.7 $\pm$ 1.4	65.0 $\pm$ 5.2
UFA/SFA	0.49	1.87	2.10	1.86

tion of the SFA/UFA ratio was observed in M cultures when ajoene was added to them.

Azoles (ketoconazole, itraconazole and saperconazole) (San-Blas *et al.*, 1993a) are very effective growth inhibitors against *P. brasiliensis*, indicating that the fungus requires specific 4,14-desmethyl sterols of the ergosta type for growth. The neutral lipid fraction of *P. brasiliensis*, Y phase, contained ergosta-5,22-dien-3 β -ol (82%) as the main free sterol, plus much lower amounts of lanosterol (12%) and ergosterol (6%). In the M phase, the predominant sterol was ergosterol (88%), accompanied by lower levels of ergosta-5,22-dien-3 β -ol (12%). These results contrast with previous ones in five strains of *P. brasiliensis* (Hamdam *et al.*, 1993) in which ergosterol and squalene comprised the bulk of sterol fraction. The presence of ergosta-5,22-dien-3 β -ol instead of ergosterol in *P. brasiliensis* (Y phase > M phase), though unusual, should only lead to minor differences in the properties of the plasma membrane, in contrast with the abrupt changes provoked when cholesterol, rather than ergosterol, is incorporated. *T. cruzi*, another polymorphic micro-organism, had ergosterol and its 24-ethyl analogue in the membranes of the epimastigote phase, while amastigotes modified these sterols with the exclusion of the 5,22 double bonds (J. A. Urbina and

others, unpublished results). In *C. albicans*, enzymic defects in ergosterol-deficient (*erg*) mutants lead to the accumulation of intermediates such as ergosta-7-en-3 β -ol or ergosta-7,22-dien-3 β -ol which were able to sustain the growth of *erg* mutants without any auxotrophic requirement for sterols. However, the depletion of ergosterol resulted in reduced assimilation of glycerol, DL-lactic acid, L-sorbose and other compounds, and to decreased amino acid transport activity (Mishra *et al.*, 1992). Whether this is the case with both phases of *P. brasiliensis* remains to be studied. In contrast with the marked effects of ajoene on the phospholipid headgroup and fatty acid composition, no significant effects were observed in the sterol composition of either phase in the presence of ajoene.

In conclusion, the antiproliferative effects of ajoene in *P. brasiliensis* were associated with a marked reduction in the content of PC, with a concomitant increase in the levels of its precursor PE and a large increase in the amounts of unsaturated fatty acids in the Y phase. This may provide an explanation for the observed alteration of the physical properties of the plasma membrane (San-Blas *et al.*, 1989) and could also lead to modifications in the activities of key membrane enzymes. These biochemical effects are remarkably similar to those reported

previously for the protozoon *T. cruzi* (Urbina *et al.*, 1993), suggesting a common antiproliferative mechanism of ajoene against lower eukaryotes and resulting in a novel mechanism of antifungal action.

ACKNOWLEDGEMENTS

To Dr Rafael Apitz-Castro for kindly providing samples of ajoene. This project was partially financed by the World Bank/World Health Organization/PNUD Special Programme of Research and Training on Tropical Diseases grant OMS/TDR 930161 (J.A.U.).

REFERENCES

- Adetumbi, M., Javor, G. T. & Lau, B. H. S. (1986). *Allium sativum* (garlic) inhibits lipid synthesis by *Candida albicans*. *Antimicrob Agents Chemother* 30, 499–501.
- Ames, B. N. & Dubin, D. T. (1960). The role of polyamines in the neutralization of deoxyribonucleic acid. *J Biol Chem* 235, 769–775.
- Apitz-Castro, R., Cabrera, S., Cruz, M. R., Ledezma, E. & Jain, M. K. (1983). Effect of garlic extracts and of three pure components isolated from it on human platelet aggregation, arachidonate metabolism, release reaction and platelet ultrastructure. *Thromb Res* 42, 155–169.
- Apitz-Castro, R., Ledezma, E., Escalante, J., Jorquera, A., Piñate, F., Moreno-Rea, J., Carrillo, G., Leal, O. & Jain, M. K. (1988). Ajoene [(E,Z)-4,5,9-trithiadodeca-1,6,11-triene 9-oxide] reversibly prevents platelet activation in dogs under extracorporeal circulation. *Arzneim-Forsch* 38, 901–904.
- Cuzner, M. L. & Davison, A. N. (1967). Quantitative thin layer chromatography of lipids. *J Chromatogr* 27, 388–397.
- Ghannoum, M. A. (1988). Studies on the anticandidal mode of action of *Allium sativum* (garlic). *J Gen Microbiol* 134, 2917–2924.
- Ghannoum, M. A., Janini, G., Khamis, L. & Radwan, S. S. (1986). Dimorphism-associated variations in the lipid composition of *Candida albicans*. *J Gen Microbiol* 132, 2367–2375.
- Goyal, S. & Khuller, G. K. (1994). Structural and functional role of lipids in yeast and mycelial forms of *Candida albicans*. *Lipids* 29, 793–797.
- Hamdan, J. S., Resende, M. A., Cunha, A. L., Alves, C. H. & Cisalpino, E. O. (1993). Partial biochemical characterization of five *Paracoccidioides brasiliensis* strains. *Curr Microbiol* 27, 91–95.
- Lacaz, C. S. (1994). *Paracoccidioides brasiliensis*: morphology, evolutionary cycle; maintenance during saprophytic life; biology, virulence, taxonomy. In *Paracoccidioidomycosis*, pp. 13–25. Edited by M. Franco, C. S. Lacaz, A. Restrepo-Moreno & G. Del Negro. Boca Raton: CRC Press.
- Larralde, G., Vivas, J. & Urbina, J. A. (1988). Concentration and time-dependence of the effects of ketoconazole on growth and sterol synthesis by *Trypanosoma (Schizotrypanum) cruzi* epimastigotes. *Acta Cient Venez* 39, 140–146.
- Manocha, M. S. (1980). Lipid composition of *Paracoccidioides brasiliensis*: comparison between the yeast and mycelial forms. *Sabouraudia* 18, 281–286.
- Mishra, P., Bolard, J. & Prasad, R. (1992). Emerging role of lipids in *Candida albicans*, a pathogenic dimorphic yeast. *Biochim Biophys Acta* 1127, 1–14.
- Restrepo, A. & Arango, M. D. (1980). *In vitro* susceptibility testing of *Paracoccidioides brasiliensis* to sulfonamides. *Antimicrob Agents Chemother* 18, 190–194.
- Restrepo, A., De Bedoutand, C. & Tabares, A. M. (1984). *In vitro* susceptibility of *Paracoccidioides brasiliensis* yeast form to antifungal agents. *Rev Inst Med Trop Sao Paulo* 26, 322–328.
- San-Blas, G. & San-Blas, F. (1994). Biochemistry of *Paracoccidioides brasiliensis* dimorphism. In *Paracoccidioidomycosis*, pp. 49–66. Edited by M. Franco, C. S. Lacaz, A. Restrepo-Moreno & G. Del Negro. Boca Raton: CRC Press.
- San-Blas, G., San-Blas, F., Gil, F., Mariño, L. & Apitz-Castro, R. (1989). Inhibition of growth of the dimorphic fungus *Paracoccidioides brasiliensis* by ajoene. *Antimicrob Agents Chemother* 33, 1641–1644.
- San-Blas, G., Calcagno, A. M. & San-Blas, F. (1993a). A preliminary study of *in vitro* antibiotic activity of saperconazole and other azoles on *Paracoccidioides brasiliensis*. *J Med Vet Mycol* 31, 169–174.
- San-Blas, G., Mariño, L., San-Blas, F. & Apitz-Castro, R. (1993b). Effect of ajoene on dimorphism of *Paracoccidioides brasiliensis*. *J Med Vet Mycol* 31, 133–141.
- Sánchez-Mirt, A., Gil, F. & Apitz-Castro, R. (1993). *In vitro* inhibitory effect and ultrastructural alterations caused by ajoene on the growth of dematiaceous fungi: *Cladosporium carrionii* and *Fonsecaea pedrosoi*. *Rev Iberoam Micol* 10, 74–78.
- Sorais-Landáez, F. & San-Blas, G. (1993). Localization of β -1,3-glucan synthetase in membranes of *Paracoccidioides brasiliensis*. *J Med Vet Mycol* 31, 421–426.
- Suutari, M. (1995). Effect of growth temperature on lipid fatty acids of four fungi (*Aspergillus niger*, *Neurospora crassa*, *Penicillium chrysogenum* and *Trichoderma reesei*). *Arch Microbiol* 164, 212–216.
- Urbina, J. A., Marchán, E., Lazard, K., Visbal, G., Apitz-Castro, R., Gil, F., Aguirre, T., Piras, M. M. & Piras, R. (1993). Inhibition of phosphatidylcholine biosynthesis and cell proliferation in *Trypanosoma cruzi* by ajoene, an antiplatelet compound isolated from garlic. *Biochem Pharmacol* 45, 2381–2387.
- Wanke, B. & Londero, A. T. (1994). Epidemiology and paracoccidioidomycosis infection. In *Paracoccidioidomycosis*, pp. 109–120. Edited by M. Franco, C. S. Lacaz, A. Restrepo-Moreno & G. Del Negro. Boca Raton: CRC Press.
- Yoshida, S., Kasuga, S., Hayashi, N., Ushiroguchi, T., Matsuura, H. & Nakagawa, S. (1987). Antifungal activity of ajoene derived from garlic. *Appl Environ Microbiol* 53, 615–617.

Received 22 October 1996; revised 26 November 1996; accepted 19 December 1996.