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Promotion of pseudomycelium formation of *Candida albicans* in culture: a morphological study of the effects of miconazole and ketoconazole

M. BORGERS
D.Sc.

M. DE BRABANDER
D.Sc.

H. VAN DEN BOSSCHE

J. VAN CUTSEM

Janssen Pharmaceutica, Research Laboratories, B-2340 Beerse, Belgium

Summary

The effects of miconazole and its new derivative ketoconazole on *Candida albicans* have been evaluated by light and electron microscopy.

The growth characteristics and morphology of *C. albicans* in culture for various periods of time in a solution consisting of Eagle's minimum essential medium supplemented with amino acids and fetal calf serum are emphasized. This medium, normally used for culturing mammalian cells, promotes a rather fast growth of *C. albicans* and favours the development of pseudomycelium. The obvious interest in using such culture conditions for drug evaluation is the prevalence of pseudomycelium, which *in vivo* is the predominant pathological form of *C. albicans*. Suppression of pseudomycelium formation is found in the 10^{-9} to 10^{-7} M concentration range of the imidazoles. Growth retardation and the destruction of both yeast and pseudomycelial forms brought about by incubating the cells with 10^{-9} to 10^{-4} M of the drugs are reported. At low doses these changes include the alteration of cell division, an increase in cell volume and a progressive deterioration of subcellular organelles at the cell periphery. At higher doses the involvement of all other organelles is observed finally leading to complete cell necrosis.

Introduction

Existing *in vitro* culture models in which the effects of antifungal drugs have been evaluated on *Candida albicans* apply almost entirely to the yeast form of this species. The prevailing form found in infectious diseases of *C. albicans*, however, is the pseudomycelial form. Hence, the availability of cultures in which pseudomycelium predominates would be of considerable interest. They not only mimic the clinical picture better, but also open the way to several new experimental approaches in relation to drug application

In this respect, the following drug effects can be evaluated in a situation which is free from host interference: (1) the minimal concentration that inhibits outgrowth of pseudomycelium from the inoculated yeast form as assessed by light microscopy; (2) the dose-related effects on growth and morphological, biochemical and cytochemical characteristics; (3) the effects of drugs on already established pseudomycelia. The latter makes it possible to differentiate fungistatic and fungicidal properties of the compounds on the vegetative form; (4) the effect of serum on drug activity; (5) the differential toxicity of drugs on mammalian cells and *C. albicans* in mixed cultures.

This paper deals with a first series of experiments on *C. albicans*, cultured in a pseudomycelium-promoting medium. The effects of different doses of miconazole and its newly synthetized analogue ketoconazole (*cis*-1-acetyl-4-[4-{[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}phenyl]piperazine) are evaluated by light and electron microscopy. Ketoconazole has recently proved to be an antifungal agent with oral activity against a broad range of yeasts and fungi (Thienpont *et al.*, 1979).

Materials and methods

C. albicans, strain B 12377 was maintained on Sabouraud agar slants at 25°C. Yeast cells were grown at 37°C on Sabouraud agar and a loopful from a 24 hr's slant was inoculated in 4·5 ml of Sabouraud broth and cultured at 37°C for 24 hr. A 0·5-ml aliquot of this culture was inoculated in 100 ml of a casein hydrolysate yeast extract glucose medium (CYG medium). Cells were grown at 37°C aerobically in a reciprocating shaker for 64 hr. From this culture a 0·2-ml aliquot was again inoculated in 100 ml CYG medium and grown for another 24 hr at 37°C while shaking (Van den Bossche *et al.*, 1978). The aim of this procedure was to prepare the cells in a standardized way, before inoculating the experimental media.

The pseudomycelium growth-promoting medium

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consisted of Eagle's minimal essential medium (Flow Laboratories) supplemented with non-essential amino acids (Flow), 10% fetal calf serum and sodium bicarbonate 2 g/l. Yeast cells were seeded at densities of $1{\cdot}5 \times 10^5$/ml or $1{\cdot}5 \times 10^4$/ml in sterile Petri dishes (diameter 9 cm) containing 10 ml of medium. Cells were grown in a humidified atmosphere of 5% CO_2 in air at 37°C.

The effects of miconazole and ketoconazole were evaluated in 2 experimental conditions. In a first series the compounds were added immediately after inoculation of the yeast cells in order to study the effects on the formation of pseudomycelium and the morphology of the yeast and pseudomycelial cells. In a second series, the compounds were added after 24 hr of growth when pseudomycelium was fully developed and yeast forms were almost totally lacking. In both experiments the cultures were exposed to miconazole and ketoconazole in doses ranging from 10^{-9} M ($\pm$ 0·5 ng/ml) to 10^{-4} M ($\pm$ 50 μg/ml). The solvent for miconazole was dimethylsulphoxide (final concentration 0·5%); ketoconazole was added as an aqueous solution. Pseudomycelium development was observed by light microscopy at various periods after inoculation. Transmission and scanning electron microscopy of the cultures was performed after 24 hr of exposure to the drugs. After harvesting, cells of both experiments were pelleted, fixed and prepared for electron microscopy as described by Borgers and De Nollin (1974).

Results

Development and growth of pseudomycelium

The inoculated yeast cells went through a very short lag phase and within one hour they had formed germ tubes. Branching hyphae occurred after 4 hr of inoculation and mycelial colonies could be observed from 8 hr onwards. They continued to grow without signs of cellular necrosis until 48 hr after inoculation.

Light and electron microscopic assessment of pseudomycelium inhibition

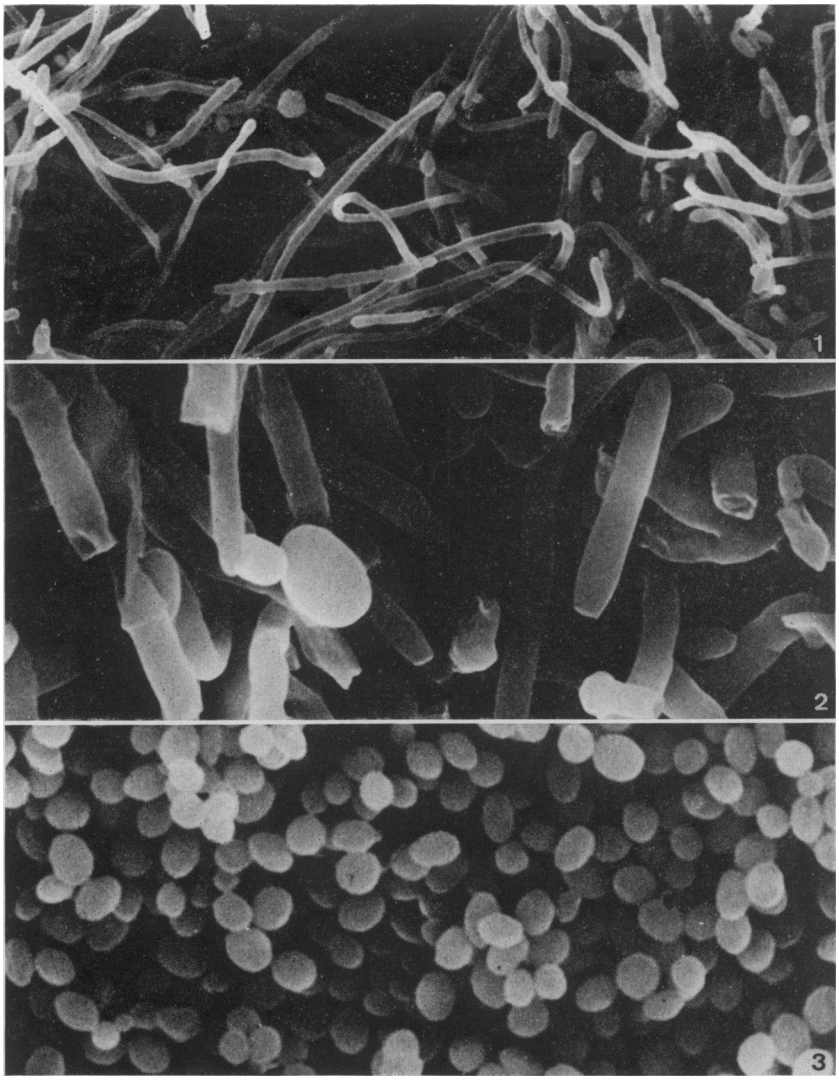
When the drugs were simultaneously added with the inoculum, ketoconazole completely inhibited the formation of pseudomycelial outgrowth from the inoculated yeast at 10^{-8} M (5·3 ng/ml) whereas miconazole had the same effect at 10^{-6} M (0·48 μg/ml).

In the scanning electron microscope the first prominent changes were seen with 10^{-9} M ketoconazole and 10^{-8} M miconazole. At variance with control cultures which almost exclusively contain very long pseudohyphae (Fig. 1), such treated cultures showed predominantly short pseudohyphae (approx. 10% of the control length) (Fig. 2). At 10^{-8} M ketoconazole (Fig. 3) and 10^{-6} M miconazole short germ tubes became rare and abnormal configurations were observed at sites of attempted budding. Such structures were usually swollen (diameter 4 μm) and protruded from abnormally swollen and scarred yeasts. Practically all cells (> 99%) were yeast forms, most of them clustered and of heterogeneous size (diameter 2–5 μm). Surface irregularities were more pronounced after exposure to the drugs at concentrations up to 10^{-5} M and many cells then had a diameter of 5–7 μm. With 10^{-4} M ketoconazole, most of the cells were smooth surfaced and only a few were swollen. On the contrary, with 10^{-4} M miconazole, many of the remaining cells were irregularly contoured and angular in shape, and possessed multiple bud scars or scars of attempted budding.

With transmission electron microscopy no differences were seen in the ultrastructure of subcellular organelles from pseudomycelium of control cultures and those after treatment with 10^{-9} M ketoconazole or 10^{-8} M and 10^{-7} M miconazole. The ultrastructure details of pseudomycelium were equally well preserved as those of the yeast cells reported in earlier studies (Borgers and De Nollin, 1974). At both seeding densities all cells were viable after 24 hr of growth in the control cultures. The lowest doses that completely inhibited pseudomycelial outgrowth (10^{-8} M for ketoconazole and 10^{-6} M for miconazole) also produced the earliest alterations on the *C. albicans* yeast form. These changes mainly consisted in an increase in volume, caused partially by hypertrophy of the cytoplasm and partially by the considerable thickening of the cell wall, and in the deposition of osmiophilic material, possibly altered phospholipids, at the cell wall. Cell necrosis was rare with these doses, but became gradually more obvious with ketoconazole from 10^{-7} M onwards and with miconazole from 10^{-5} M onwards. Swollen cells with multiple bud scars were numerous and had a very thick wall in which many phospholipid-like vesicles and whorls settled. It was most unusual that the ketoconazole-treated cells and to a lesser extent the miconazole-treated ones contained abundant lipid globules and some of them membrane-associated needle-like crystals. At the 10^{-4} M concentration, most cells of the ketoconazole cultures appeared to be completely necrotic and were filled with lipid globules. With miconazole, a great number of cells were lysed in which the organelles were only just recognizable but a large portion showed lesions comparable to the 10^{-5} M-treated cells.

Effects of the drugs on the morphology of outgrown pseudomycelium

In this series of experiments the inoculated yeast cells were grown for 24 hr to develop into pseudomycelia and were then exposed to the drugs for another 24 hr. After 48 hr of growth the control



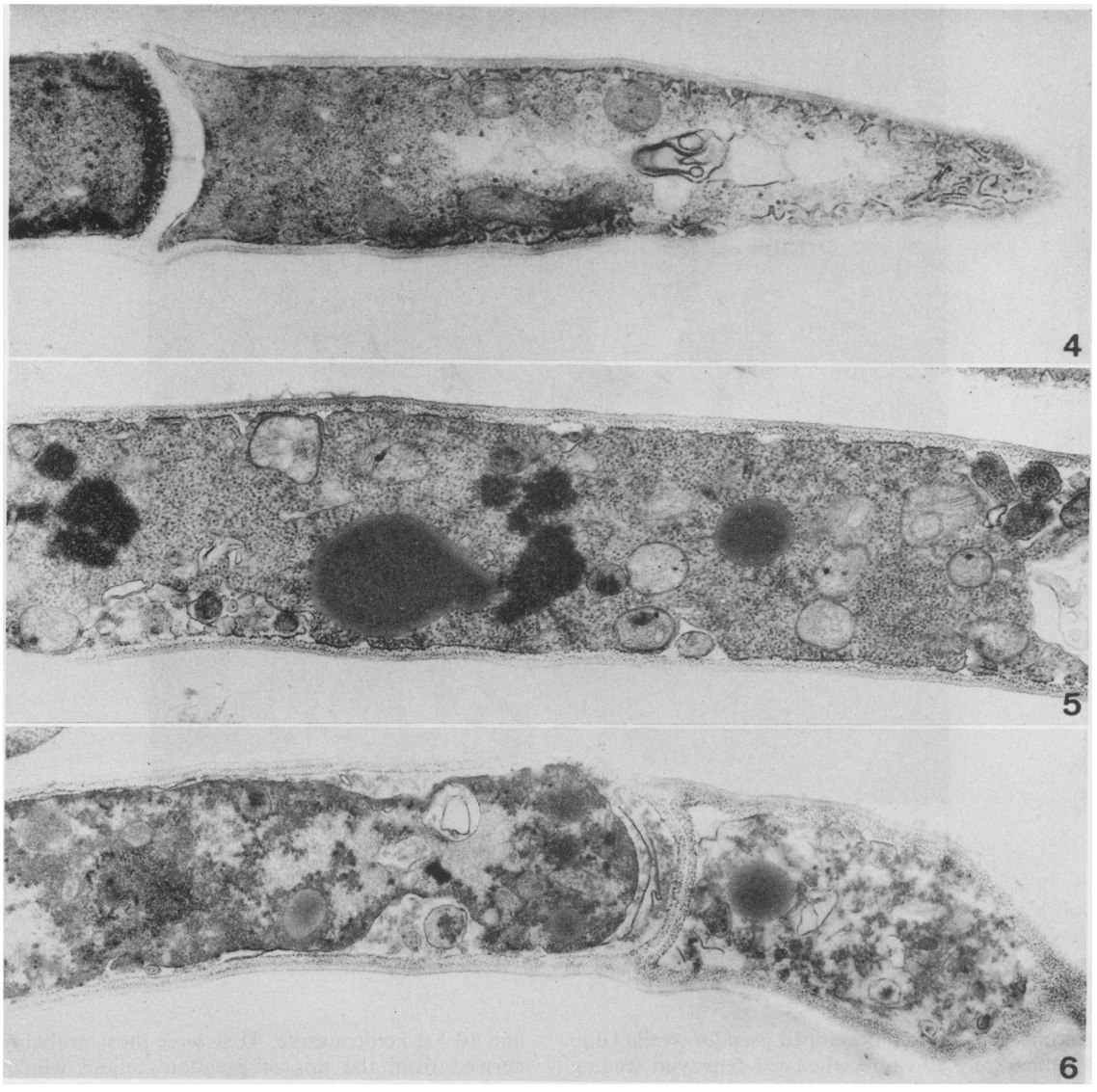
FIGS 1–3. Scanning electron micrographs.

1. Control culture of *C. albicans* grown for 24 hr in the pseudomycelium-promoting medium. Practically all cells consist of long, branching hyphae (× 720)
2. Culture exposed to 10^{-8} M (4·8 ng/ml) miconazole for 24 hr. The culture consists of short non-branching pseudomycelial elements and few yeast cells (× 2800)
3. Culture exposed to 10^{-8} M (5·3 ng/ml) ketoconazole for 24 hr. Practically all cells are in the yeast form, germ tubes are rare and no branching hyphae are noted (× 1210)

cultures showed well developed pseudomycelia (Fig. 4) which were all viable when cell density at seeding was kept at $1{\cdot}5 \times 10^4$ cells/ml. Miconazole at 10^{-8} to 10^{-7} M and ketoconazole at 10^{-9} M did not provoke morphologically detectable changes when added after 24 hr of inoculation. Very thick walled yeast-type cells which were sometimes elongated, showed osmiophilic deposits after 10^{-6} M miconazole and 10^{-8} M ketoconazole. They were most probably derived from the tips of pseudomycelium which, under the influence of the drugs, were no longer able to produce hyphae but instead allowed the outgrowth of altered yeast forms. Such cells became more abundant at 10^{-5} M miconazole and 10^{-6} M ketoconazole. These doses equally produced proliferation of unusually large peroxisomes in

yeasttype cells. Enlarged peroxisomes were not seen pseudomycelium. At concentrations $\geqslant 10^{-5}$ M, both in drugs induced necrosis of outgrown pseudomycelium. The necrotized cells always possessed a more or less intact cell wall without osmiophilic inclusions. At the 10^{-4} M concentration, miconazole appeared to be at least as toxic to outgrown pseudomycelia as ketoconazole (Figs 5 and 6).

Scanning electron microscopy has confirmed these structural alterations as far as transformation of hyphae to abnormal yeast forms is concerned. When doses were applied in a range that still allowed retarded but continuous growth (10^{-9} M to 10^{-6} M of both drugs) a dose-dependent return to the yeast form was seen. Such yeasts were voluminous and irregularly surfaced. Although transmission electron



FIGS 4–6. Transmission electron micrographs of cultures grown for 48 hr in the pseudomycelium-promoting medium.
4. Control culture showing the ultrastructure of a hypha (× 19 500)
5. Culture of *C. albicans* grown for 24 hr and then exposed to 10^{-4} M (53 μg/ml) ketoconazole for 24 hr. Note the deterioration of the subcellular organelles in the hyphal cytoplasm (× 17 000)
6. Culture grown for 24 hr and exposed to 10^{-4} M (48 μg/ml) miconazole for 24 hr. The cytoplasm is totally necrotic (× 22 000)

microscopy revealed that from 10^{-5} M onwards many of the outgrown pseudomycelia were necrotic, their surface morphology in the scanning microscope appeared to be normal.

Discussion

Although pseudomycelium is the predominating form in candidiasis, practically all *in vitro* studies involved with the evaluation of antifungal drugs on *C. albicans* have been performed on the yeast form. These include the determination of the minimal inhibitory and the fungicidal concentration and studies on the mechanism of action and drug resistance. This is mainly because of the lack of suitable conditions for inducing and maintaining pure pseudomycelial development in culture. A few years ago, Lee, Buckley and Campbell (1975) succeeded in producing purely mycelial phase cells cultivated in a liquid synthetic medium. However, the transformation was transitory so that the cultures only contained pure pseudomycelium between 18 and 27 hr after inoculation. A long-lasting viable culture of pseudomycelium, closely resembling the form found in many superficial and systemic infections, is needed clearly to evaluate the efficacy of drugs on this phase of *C. albicans*. Such conditions were produced in the present study and offer the possibility of experimental approaches that may contribute to the understanding of pathogenicity and therapy of this form. First of all, the presence of serum which definitely favours this long-lasting pseudomycelial growth makes it likely the better to mimic the *in vivo* situation. Secondly, since this medium is a normal growth-supporting medium for mammalian cells, the interaction between host and invasive cells can be studied.

The present results of the simultaneous exposure of inoculated cells to miconazole and ketoconazole for 24 hr reveal that ketoconazole is about 100 times more potent in inhibiting pseudomycelium formation and provoking subcellular changes than miconazole under the same conditions. This is at variance with the effects of these drugs on *C. albicans* cultured in common yeast-promoting media. In an earlier report, Van den Bossche, Willemsens and Cornelissen (1977) described the differential sensitivity towards drugs as being strongly dependent on growth conditions. Furthermore, Van Cutsem *et al.* (unpublished results) found that the addition of bovine serum to Sabouraud culture medium changes the effects of ketoconazole and miconazole.

In contrast to studies based on the yeast form of *C. albicans* when the drug is added to well developed and still growing pseudomycelium (a situation very much resembling that when a curative treatment is imposed) electron microscopy confirms the high potency of both miconazole and ketoconazole in killing an existing pseudomycelium. The morphological observations presented accord with previous ultrastructural findings (De Nollin and Borgers, 1974) and concern the sequential and dose-dependent changes at the cell periphery, the increase in cell volume, the increase in the number of peroxisomes, the deterioration of the substructural organelles such as nuclei, mitochondria and the vacuolar system, fatty degeneration of the cytoplasm and, finally, plasmolysis.

Although this first series of experiments on pseudomycelium cultures of *C. albicans* gives a reasonable impression of what can be achieved with this *in vitro* model, more information is needed from exposing cultures to antifungals at different phases of growth for longer periods of time and thereby imitating even more closely the usual treatment schedule as applied in *C. albicans* infections. As well as evaluating its activity on the more conventional yeast forms, assessing the effect of a drug on pseudomycelium enables one to differentiate between one which is a true fungicide and one which is merely fungistatic.

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References

BORGERS, M. & DE NOLLIN, S. (1974) The preservation of subcellular organelles of *Candida albicans* with conventional fixatives. *Journal of Cell Biology*, **62**, 574.

DE NOLLIN, S. & BORGERS, M. (1974) The ultrastructure of *Candida albicans* after *in vitro* treatment with miconazole. *Sabouraudia*, **12**, 341.

LEE, K.L., BUCKLEY, H.R. & CAMPBELL, C. (1975) An amino acid synthetic medium for the development of mycelia and yeast forms of *Candida albicans*. *Sabouraudia*, **13**, 148.

THIENPONT, D., VAN CUTSEM, J., VAN GERVEN, F. & JANSSEN, P.A.J. (1979) Ketoconazole – A broad spectrum orally active antimycotic. *Experientia*, **35**, 606.

VAN DEN BOSSCHE, H., WILLEMSEMS, G., COOLS, W., LAUWERS, W.F.J. & LE JEUNE, L. (1978) Biochemical effects of miconazole on fungi. II. Inhibition of ergosterol biosynthesis. In: *Candida albicans. Chemicobiological Interactions*, **21**, 59.

VAN DEN BOSSCHE, H., WILLEMSENS, G. & CORNELISSEN, F. (1977) The *in vitro* action of R 41 400 and miconazole nitrate on the growth of *Candida albicans*. Janssen Pharmaceutica, *Preclinical Research Report*, R 41 400/8, acc. No. 13373.