

## The In vitro Effect of Fluconazole on the Filamentous Form of *Pityrosporum ovale*

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The antimycotic activity of fluconazole against the filamentous form of *Pityrosporum ovale* was studied in vitro. *P. ovale* was grown on human stratum corneum in vitro with and without the addition of different concentrations of fluconazole. In control cultures hyphae were produced in 25% of the cells compared to only 4% after exposure to fluconazole 1 µg/ml. In control cultures 16% of the fungal cells showed signs of necrosis, due to the normal turnover rate of the cells, compared to 65% of the fungal cells exposed to 1 µg/ml of fluconazole. In the transmission electron microscope the typical thick-walled fungal cells with their characteristic budding were observed in control cultures. However, after exposure to 1 µg/ml of fluconazole the *P. ovale* cells showed extensive signs of necrosis, with loss of internal organelles and disinterruption of the cell wall. The results obtained in this in vitro model mimic the in vivo situation in pityriasis versicolor. There is a parallel between the good results obtained in this system and the good clinical effect of fluconazole in *Pityrosporum*-related diseases.

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Fluconazole is a synthetic, orally active, bis-triazole derivative with broad antifungal action which inhibits the synthesis of ergosterol (1–3).

The lipophilic yeast *Pityrosporum ovale* is a member of the normal human cutaneous flora (4). However, it is not only a saprophyte but it is also associated with several diseases such as pityriasis versicolor, *Pityrosporum* folliculitis, seborrhoeic dermatitis and some forms of atopic dermatitis, psoriasis and confluent and reticulate papillomatosis (4). In pityriasis versicolor the lipophilic yeast *P. ovale* changes from the round blastospore form to the mycelial form. The antifungal activity may be different for the spore form and the hyphal form of the organism.

The aim of the present investigation was to study the effect of fluconazole on both the hyphal and spore form of *P. ovale* in vitro.

### MATERIALS AND METHODS

#### Stratum corneum preparation

The method for obtaining stratum corneum has previously been described (5, 6). Full thickness skin samples were obtained from plastic surgery on female breasts. Epidermis was peeled off from full thickness skin by gently heating the skin sample to 65°C. The heating causes a split at the basement membrane level. The stratum corneum was isolated by incubating pieces of epidermis in a 0.1% aqueous trypsin solution at 37°C for 2 h. This disrupts the desmosomes and the detached epidermal cells could easily be washed off, leaving the stratum corneum intact. Stratum corneum was then sterilized with ethylene oxide (gas) and kept in a freezer at –70°C until use.

#### Fungi

The strain used in this study was *P. ovale* ATCC 44341. It was grown on a glucose-neopeptone-yeast extract agar medium with the addition of glycerol monostearate (2.5 g/l), Tween 80 (2 ml/l) and olive oil (20 ml/l) at 37°C for 4 days before use.

#### Experimental design

The model for filamentous growth of *P. ovale* has previously been described. Pieces of stratum corneum (1 cm<sup>2</sup>) were placed on top of the same culture medium used for culture of the yeast. Fluconazole, kindly provided by Pfizer in Sweden, was dissolved in dimethyl formamide to give a stock solution containing 1,000 µg/ml of the drug. Further dilutions were made in phosphate-buffered saline (PBS) (pH 7.2). Immediately after inoculation on the culture medium, the stratum corneum samples were exposed to 0.1 ml of a freshly made solution containing a mixture of *P. ovale* (10<sup>8</sup> cells/ml) and fluconazole in concentrations of 0.01, 0.1 and 1 µg/ml. Control solutions were made with *P. ovale* and PBS. The culture plates were then incubated in a microaerophilic environment at 37°C for 6 days. All samples were made in duplicate.

**Light microscopy (LM).** After 6 days of incubation half of the stratum corneum pieces from all samples were placed on a glass slide, stained by the periodic acid Schiff reaction (PAS) for light microscopy examination. Twenty-five different fields (× 1,000) were examined and the mean number of the percentage of cells which produced hyphae or showed signs of necrosis was estimated for the various concentrations of fluconazole and controls.

**Transmission electron microscopy (TEM).** At the end of the experiment the other half of all stratum corneum samples were immediately fixed at room temperature in 3% glutaraldehyde buffered to pH 7.4 with 0.1 M sodium cacodylate for several days and rinsed in the same buffer supplemented with 4% sucrose. Samples were postfixed in 1% OsO<sub>4</sub> buffered to pH 7.4 with 0.1 M sodium cacodylate at room temperature for 1 h. Thereafter, the samples were rinsed in the same buffer and dehydrated in graded concentrations of ethanol. Each sample was then embedded in Epon. Ultrathin sections of the cells were stained with uranyl acetate and lead citrate prior to examination in a Philips EM 400 transmission electron microscope.

### RESULTS

**Light microscopy.** Table I shows the light microscopic observations after incubation of *P. ovale* cells with various concentrations of fluconazole. Incubation with fluconazole reduced the number of hyphae being produced, and with higher concentrations of fluconazole a high number of fungal cells showed signs of necrosis.

**Transmission electron microscopy.** In control cultures the majority of the fungal cells looked healthy, with a uniformly thick electron-dense cell wall, and the organelles within the cells were clearly outlined (Fig. 1). Fungal cells were often seen in close contact with stratum corneum cells. Around 25% of the cells produced hyphal elements. A small proportion of the cells showed signs of necrosis due to the normal turnover of the cells. With increasing concentration more and more cells showed necrotic changes with disintegration of internal organelles and disruption of the cell wall, and with the highest



Fig 1. Transmission electron microscopy of *Pityrosporum ovale* from a control culture showing the typical thick cell wall and type of budding ( $\times 48,000$ ).



Fig 2. Transmission electron microscopy showing extensive necrotic changes with disruption of organelles and disintegration of the cell wall in *Pityrosporum ovale* after exposure to fluconazole ( $\times 37,000$ ).

concentration of fluconazole ( $1 \mu\text{g/ml}$ ) several cells were more or less empty and with severe disintegration of the cell wall, (Fig. 2).

#### DISCUSSION

We have previously described in detail a model for production of hyphae in *P. ovale* on human stratum corneum in vitro (5,

Table I. The effect of fluconazole on *Pityrosporum ovale* on human stratum corneum in vitro

	% hyphae	% necrotic cells
Control	25	16
Fluconazole:		
0.01 $\mu\text{g/ml}$	14	17
0.1 $\mu\text{g/ml}$	9	23
1 $\mu\text{g/ml}$	4	65

6). The activity of ketoconazole and itraconazole against *P. ovale* has been investigated with this model (7). The production of hyphae was reduced from 26 to 3% of cells with ketoconazole ( $1 \mu\text{g/ml}$ ), and with itraconazole ( $1 \mu\text{g/ml}$ ) the number of cells that produced hyphae was reduced from 26 to 10% (7). In pityriasis versicolor *P. ovale* changes from the round or oval blastospore form, to the mycelial form and this in vitro model for investigating the activity of antifungal drugs against *P. ovale* is much closer to the in vivo situation than conventional testing for minimal inhibitory concentration (MICs) of antimicrobial drugs.

In the present investigation, fluconazole could block the production of hyphae in a concentration that was lower than the concentration for MIC (8). With a concentration of only  $0.01 \mu\text{g/ml}$  of fluconazole the number of *P. ovale* cells that produced hyphae was reduced from 25% to 14%, and with a concentration of  $1 \mu\text{g/ml}$  of fluconazole only 4% of the fungal cells produced hyphae. An increase in the number of fungal cells that showed sign of necrosis was seen with a concentration of fluconazole of  $0.1 \mu\text{g/ml}$ . The number was increased from 16% in the control culture to 23%, and with  $1 \mu\text{g/ml}$  of fluconazole 65% of the *P. ovale* cells showed signs of necrosis. The TEM fully documented the effect of fluconazole on *P. ovale* with disintegration of internal organelles and disruption of the cell wall.

The good clinical effect of fluconazole in the treatment of pityriasis versicolor is explained, as documented in this study, by the high activity of this drug against *P. ovale* (1, 9). The concentration of fluconazole in the skin, especially in the stratum corneum, after oral medication with just one single 150 mg capsule is more than a 100 times above the concentration that blocks the production of hyphae and induces necrosis in a high number of *P. ovale* cells.

In conclusion, this model for production of hyphae and growth of *P. ovale* on human stratum corneum in vitro is very valuable for screening the activity of antifungal drugs thought to be effective in *Pityrosporum*-related diseases.

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