

## INVESTIGATIVE REPORT

# *In vitro* Activity of R126638 and Ketoconazole Against *Malassezia* Species

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The *in vitro* activity of a new triazole R126638 against *Malassezia* yeasts was compared with that of ketoconazole. With the agar dilution technique, minimal inhibitory concentrations were lower for R126638 compared with ketoconazole against *Malassezia globosa*, *M. obtusa*, *M. slooffiae*, *M. restricta* and two strains of *M. sympodialis*. On human stratum corneum *in vitro*, both R126638 and ketoconazole were very effective in reducing the production of hyphae from 15% to 1% with R126638 and to 2% with ketoconazole. Scanning electron microscopy did not reveal obvious surface differences between untreated cultures and cultures exposed to ketoconazole or R126638 in the concentration range 0.01–1 µg/ml. However, transmission electron microscopy showed partial to complete necrosis of the cytoplasmic organelles of *Malassezia* yeasts. The combined scanning electron microscopy and transmission electron microscopy findings confirm earlier observations of the “mummifying” effect of azoles against *Malassezia* spp. In conclusion, R126638 is an interesting new triazole with high activity against the *Malassezia* yeasts, which are involved in pityriasis versicolor and seborrhoeic dermatitis. **Key words:** *Malassezia* species.; R126638; ketoconazole; MIC; hyphae formation.

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R126638 is a broad-spectrum antifungal belonging to the class of triazoles (1, 2). It has excellent potential for oral and topical treatment of fungal infections of the skin, hair, nails, oral and genital mucosa. *In vitro* data has demonstrated R126638 activity against dermatophytes (*Trichophyton* spp., *Microsporum canis*, *Epidermophyton floccosum*), yeasts (*Candida* spp. and *Malassezia* spp.) and many other fungi. Furthermore, animal experiments have provided evidence for a potent therapeutic effect of R126638 that has proved to be 4- to 8-fold superior to that of itraconazole, especially for superficial fungal infections (1). The strong activity of R126638 is ascribed to its prominent affinity for fungal cytochrome P450, which is involved in the biosynthesis of ergosterol from

lanosterol. Ergosterol is a vital cell membrane component in fungi, and its inhibition by R126638 leads to abated fungal growth (3).

Activity of R126638 against several dermatophytes and *M. globosa* was demonstrated in the stratum corneum of healthy volunteers taking 100 mg or 200 mg R126638 daily for one week (4).

Ketoconazole is a broad-spectrum, orally and topically active imidazole derivative (5). It has a high activity against *Malassezia in vitro* and is also effective in the treatment of various *Malassezia*-associated diseases.

The genus *Malassezia* can be divided into seven species (6). *M. furfur*, *M. sympodialis*, *M. globosa*, *M. obtusa*, *M. restricta* and *M. slooffiae* are the lipophilic members of the genus. *M. pachydermatis* is the non-lipophilic member of the genus and isolated primarily from animals.

The lipophilic *Malassezia* species are members of the normal skin flora (7, 8). However, they are not only harmless saprophytes but also opportunistic pathogens. They are the aetiological agent or play an important role in pityriasis versicolor, *Pityrosporum (Malassezia)* folliculitis, seborrhoeic dermatitis and atopic dermatitis (7, 9, 10). Even systemic infections with these yeasts have been reported (8).

In a paper by Gupta et al. (11), the *in vitro* susceptibility (minimal inhibitory concentrations, MIC) of the seven different *Malassezia* species to azoles (ketoconazole, itraconazole and voriconazole) and allylamine (terbinafine) was tested. All of the *Malassezia* yeasts were highly sensitive to the three azole drugs. However, with terbinafine only *M. pachydermatis* and *M. sympodialis* were highly sensitive. For the other five *Malassezia* species the susceptibility to terbinafine was very low for several isolates.

In pityriasis versicolor the lipophilic *Malassezia* yeasts change from the round yeast cells to the hyphal form. The antifungal activity may be different for the yeast cell and the hyphal form of the organism. In earlier studies we have been able to produce hyphae in *Malassezia* on human stratum corneum *in vitro* (12). The yeast was at that time named *P. ovale*. However, the strain with the highest production of hyphae has later been typed to *M. sympodialis*. It produced hyphae in 21% of the cells and another *M. sympodialis* strain produced hyphae in 19% of the cells. The

activity of ketoconazole and itraconazole (13) as well as fluconazole (14) against *Malassezia* has been studied earlier in this model. In these studies ketoconazole (1 µg/ml) and itraconazole (1 µg/ml) reduced the number of hyphae from 26% to 3% with ketoconazole and to 10% with itraconazole. In the study with fluconazole the production of hyphae was reduced from 25% to 4% when fluconazole (1 µg/ml) was added to the solution with *M. sympodialis*.

The aim of the present study was to determine: (i) the MIC of R126638 and ketoconazole against the various *Malassezia* species; and (ii) to investigate the *in vitro* effect of R126638 and ketoconazole against both the hyphal and yeast cell forms of *M. sympodialis*.

## MATERIALS AND METHODS

Micro-organisms *M. pachydermatitis* CBS 1871, *M. furfur* CBS 7019, *M. sympodialis* CBS 7222, *M. slooffiae* CBS 7956, *M. globosa* CBS 7966, *M. obtusa* CBS 7876 and *M. restricta* CBS 7877 were included in the study. Two other strains known for their ability to produce hyphae on human stratum corneum *in vitro*: *M. sympodialis* ATCC 44031 and ATCC 44341 were also included. The strains were maintained on a modified Leeming-Notman agar (7) at 37°C.

R126638 was obtained from Barrier Therapeutics, Geel, Belgium. Ketoconazole was bought from Sigma, St Louis, USA.

### Minimal inhibitory concentrations estimated by the agar dilution technique

R126638 and ketoconazole were first dissolved in dimethylformamide (DMF) and then diluted in phosphate buffered saline (PBS, pH 7.4) to give stock solutions containing 1000 µg/ml of R126638 and ketoconazole and 20% of DMF. Other stock solutions with lower concentrations were obtained by further dilutions in PBS. From the various stock solutions, dilutions were made directly with the test culture medium, Diagnostic Sensitivity Test agar (DST) (Oxoid, UK) with the addition of Tween 80 (2 ml/l) and glycerol monostearate (2.5 g/l) to obtain concentrations of 0.005–100 µg/ml of R126638 and ketoconazole in the test medium.

*Malassezia* yeast cells were added on the agar medium, using a Pasteur pipette, in concentrations of 10<sup>7</sup> cells/ml and in a volume of 20 µl. Plates were incubated at 37°C and read after 1, 2 and 3 days. The experiment was performed in duplicate and the MIC was defined as the lowest concentration of the drugs that inhibited growth.

### In vitro effect of R126638 and ketoconazole on the filamentous form of *M. sympodialis*

The method for obtaining stratum corneum has been described in detail earlier (12). 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, making a break at the basal membrane level. The stratum corneum was obtained by incubating pieces of epidermis in a 0.1% aqueous trypsin solution at 37°C for 2 h, disrupting the desmosomes, then the detached epidermal cells could easily be washed off leaving the stratum corneum intact. Stratum corneum was stored at –70°C until use.

Pieces of stratum corneum (1 cm<sup>2</sup>) were placed directly on the culture medium (Leeming-Notman). *M. sympodialis*

(ATCC 44341) in concentrations of 10<sup>8</sup> cells/ml was mixed with R126638 or ketoconazole in concentrations of 0.01, 0.1 and 1 µg/ml and spread over the entire stratum corneum piece. The culture plates were then incubated in a micro-aerophilic environment at 37°C for 6 days. The micro-aerophilic environment was obtained and maintained using the micro-aerophilic system, gas-generating kit from Oxoid (Basingstoke, Hampshire, UK). At the end of the experiment half of the stratum corneum samples were processed for light microscopy and the others were processed for transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (13).

The stratum corneum piece was placed on a glass slide, stained by the periodic acid Schiff reaction (PAS) for light microscopy examination. Twenty-five different fields (× 1000) were examined and the mean numbers of the percentage of cells producing hyphae were estimated for the various concentrations of R126638, ketoconazole and controls (14).

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 7% sucrose. Samples were post-fixed in 1% OsO<sub>4</sub> buffered to pH 7.4 with 0.1 M sodium cacodylate at room temperature for one h. Thereafter, the samples were rinsed in the same buffer and dehydrated in graded series of ethanol. The samples were then embedded in Epon. Ultra-thin sections of the cells were stained with uranyl acetate and lead citrate prior to examination in a Philips EM 400 electron microscope (13).

## RESULTS

### Minimal inhibitory concentration determined by the agar dilution technique

The MICs for R126638 and ketoconazole are shown in Table I. R126638 was more effective than ketoconazole with MICs of 0.01 µg/ml against *M. obtusa*, *M. restricta* and all strains of *M. sympodialis*. However, there was a variation in the sensitivity of the various *Malassezia* species to both drugs. *M. sympodialis*, *M. restricta*, *M. obtusa*, *M. globosa* and *M. pachydermatitis* were the species that were most sensitive to R126638 and *M. furfur* and *M. slooffiae* were less sensitive. *M. globosa* and *M. slooffiae* were the strains with the lower sensitivity to ketoconazole.

Table I. *In vitro* activity (minimal inhibition concentrations (MIC), µg/ml) of R126638 and ketoconazole against the *Malassezia* yeasts

	MIC	
	R126638	Ketoconazole
<i>M. furfur</i> CBS 7019	0.05	0.05
<i>M. pachydermatitis</i> CBS 1871	0.02	0.02
<i>M. slooffiae</i> CBS 7956	0.05	0.1
<i>M. globosa</i> CBS 7966	0.02	0.1
<i>M. obtusa</i> CBS 7876	0.01	0.02
<i>M. restricta</i> CBS 7877	0.01	0.02
<i>M. sympodialis</i> CBS 7222	0.01	0.02
<i>M. sympodialis</i> ATCC 44031	0.01	0.02
<i>M. sympodialis</i> ATCC 44341	0.01	0.01

*In vitro* effect of R126638 and ketoconazole on the filamentous form of *Malassezia sympodialis*

Light microscopy showed that on human stratum corneum *in vitro* both R126638 and ketoconazole were very effective in reducing the production of hyphae from 15% to 1% with R126638 and to 2% with ketoconazole (Table II). Besides, with higher concentrations of both drugs, many cells were swollen and stained poorly.

Table II. Effect of R126638 and ketoconazole on the production of hyphae in *Malassezia sympodialis* revealed by light microscopy

Substance	Hyphae (%)
<b>Control</b>	15
<b>R126638</b>	
1 µg/ml	1
0.1 µg/ml	3
0.01 µg/ml	6
<b>Ketoconazole</b>	
1 µg/ml	2
0.1 µg/ml	5
0.01 µg/ml	9

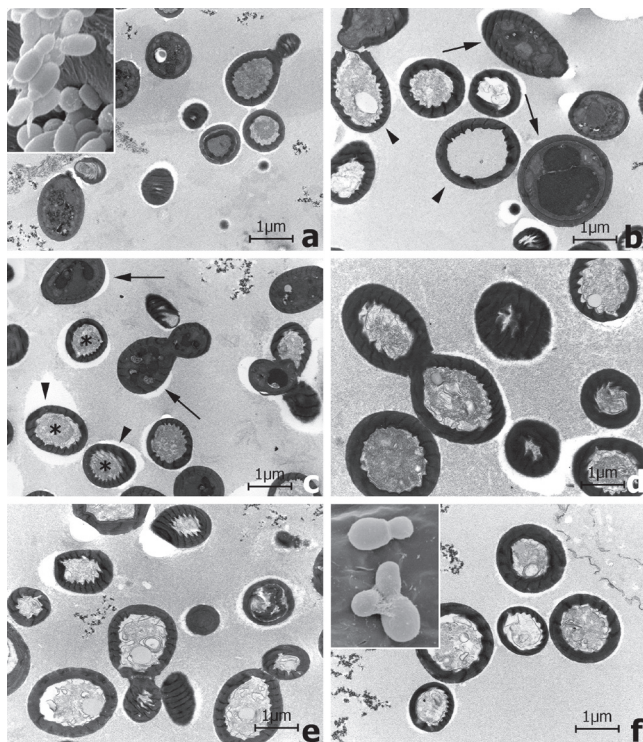


Fig. 1. Transmission and scanning (inserts) electron microscopy of *M. sympodialis*. (a) Control culture with normal yeasts showing a healthy subcellular structure. (b) R126638 0.01 µg/ml culture with normal (arrow) and affected (arrowhead) yeasts with destruction of subcellular organelles. (c) Ketoconazole 0.1 µg/ml culture with apparently normal (arrow) and affected yeasts (arrowhead) with destruction of subcellular organelles (asterisks). (d) R126638 0.1 µg/ml culture with affected cells showing necrosis. (e) Ketoconazole 1 µg/ml culture with necrosis of the yeast cells. (f) R126638 1 µg/ml culture with necrosis of the yeast cells.

SEM did not reveal obvious surface differences between untreated cultures and cultures exposed to ketoconazole or R126638 in the concentration range of 0.01 to 1.0 µg/ml. However, TEM showed partial to complete necrosis of the cytoplasmic organelles of *M. sympodialis*. The fully necrotic cells amounted to 6, 60, 76 and 100% with 0, 0.01, 0.1 and 1 µg/ml, respectively, of R126638 (Fig. 1) and to 6, 41, 62 and 97% with 0, 0.01, 0.1 and 1 µg/ml, respectively, of ketoconazole. There were no differences in morphological changes induced by the azoles between the yeast form and the few remaining budding yeasts.

## DISCUSSION

Both R126638 and ketoconazole had a high *in vitro* activity against the *Malassezia* yeasts. The two strains of *M. sympodialis* that easily produce hyphae on human stratum corneum *in vitro* were very sensitive to R126638. *M. sympodialis* and *M. globosa* are the two species that are most commonly isolated from patients with pityriasis versicolor (15), and *M. globosa* and *M. furfur* have commonly been cultured from patients with seborrhoeic dermatitis (16).

We described earlier a model that constantly enables the production of hyphae in the *Malassezia* yeasts (12). This model has been used previously to study the effect of fluconazole (14), ketoconazole and itraconazole (13) on the production of hyphae, as well as on the morphology of the *Malassezia* yeasts.

In the present study both R126638 and ketoconazole were very effective in blocking the production of hyphae in *Malassezia*. This was seen both with light- and electron microscopy. The combined SEM and TEM findings confirm earlier observations of the “mummifying” effect of azoles against *Malassezia* species. A direct necrotic effect has been obtained after azole treatment, showing complete degeneration of internal organelles, but without obvious alterations of the cell periphery. This is clearly shown by light- and electron microscopy. Such a “mummifying” effect of azoles may foster confusion about therapeutic outcome, when therapy is verified by whole mount inspection of the fungus (KOH preparation) as usually done in pityriasis versicolor (17).

R126638 is an interesting new triazole with high activity against the *Malassezia* yeasts, the yeasts involved in pityriasis versicolor and seborrhoeic dermatitis. In conclusion, this model for *in vitro* production of hyphae in *Malassezia* is valuable for screening the activity of antimycotic agents against the filamentous form of these yeasts.

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