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Antifungal activity of allylamines on *Epidermophyton floccosum:* scanning electron microscopy study

P. Butty, M. Mallié & J.-M. Bastide
Laboratoire d'Immunologie et Parasitologie, Faculté de Pharmacie, Montpellier, France

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Key words: Naftifine, terbinafine, scanning electron microscopy, *Epidermophyton floccosum*

Abstract

The action of allylamine antifungal agents on *Epidermophyton floccosum* was studied using scanning electron microscopy. After 7 days of culture on Sabouraud dextrose agar, *Epidermophyton floccosum* samples were brought in contact with concentrations of 0.2 and 2 $\mu g\,ml^{-1}$ and 0.01 and 0.1 $\mu g\,ml^{-1}$ of naftifine and terbinafine, respectively. Lesions observed after 24 h, 3 and 7 days of contact were mainly on the structure and rigidity of the mycelial and macroconidial wall. They were characterized by hyphal ballooning and twisting and by apical bulbous bulges. Deterioration of macroconidia was characterized by wall exfoliation. The intensity of the deterioration depended on the dose and only slightly on the length of time that the sample and the antifungal drug were in contact.

Mots clés: Naftifine, terbinafine, microscopie électronique à balayage, *Epidermophyton floccosum*

Résumé

L'action des allylamines sur *Epidermophyton floccosum* est étudiée en microscopie électronique à balayage. Après 7 jours de culture sur le milieu gélosé de Sabouraud, les échantillons d'*Epidermophyton floccosum* sont mis en contact avec des concentrations de 0,2 et 2 $\mu g\,ml^{-1}$ et 0,01 et 0,1 $\mu g\,ml^{-1}$ de naftifine et de terbinafine respectivement. Les lésions observées après 24 h, 3 et 7 jours de contact portent principalement sur des modifications de l'aspect morphologique des filaments mycéliens et des macroconidies. Elles sont caractérisées sur le mycélium par des ballonnements, des torsions et des apex bulbiformes. Sur les macroconidies on observe une exfoliation importante de leur paroi. L'intensité de ces lésions est dépendante de la dose et seulement faiblement dépendante du temps de contact entre l'échantillon et l'antifongique.

Introduction

The difficulty of treating dermatophyte-induced infections, particularly in AIDS patients, underscores the need to look for new molecules that will enhance the spectrum of activity, optimize routes of administration and minimize side effects. The last few years have seen the emergence of numerous antifungals which have enhanced activity against most mycoses. Among them, allylamines, a new class of synthetic antifungals, have potent activity. The spectrum of activity of naftifine and terbinafine extends to numerous fungi pathogenic for humans, among which dermatophytes are recognized as being extremely sensitive both *in vitro* and *in vivo*.

In the present study we observed morphological modifications induced by naftifine and terbinafine on *Epidermophyton floccosum*. Two antifungal concentrations were used, chosen according to their minimal inhibitory concentration. The contact time between antifungals and samples were 24 h, 3 and 7 days. Observations were made with scanning electron microscopy in order to highlight and specify the morphological modifications induced on the mycelium and conidia of *Epidermophyton floccosum* by these two antifungals.

Material and methods

Strain

The *Epidermophyton floccosum* strain (IVP 1618) came from a recent hospital sample. The strain was subcultured several times on Potatoe dextrose agar (PDA) and Borelli medium in order to facilitate the production of macroconida. It was maintained on Sabouraud dextrose agar (SDA) for 7 days at 28 °C before samples were collected for study.

Antifungal agents

A stock solution of naftifine (Schering S.A.) and terbinafine (Sandoz S.A.) were prepared at concentrations of 2000 and 100 $\mu g\ ml^{-1}$, respectively, in a dimethylsulfoxide (DMSO)-water (1:9; v/v) mixture. Dilutions were prepared in sterile distilled water in order to obtain solutions with 20 and 2 $\mu g\ ml^{-1}$ and 1 and 0.1 $\mu g\ ml^{-1}$ of naftifine and terbinafine, respectively. The test antifungal solution were prepared in Sabouraud dextrose broth (SDB) to make the two final concentrations of naftifine and terbinafine (2 and 0.2 $\mu g\ ml^{-1}$ of naftifine and 0.01 and 0.1 $\mu g\ ml^{-1}$ of terbinafine). These concentrations correspond to the MIC and ten times the MIC previously evaluated on SDA [1].

Antifungal treatment of samples

Culture samples were prepared by collecting colony fragments of 5 square millimeters from a 7-day growth on SDA. Control samples were put in contact with either the SDB or with SDB plus DMSO for 24 h, 3 and 7 days. Treated samples were maintained in contact with the antifungals at each of the 2 concentrations selected for 24 h, 3 and 7 days under agitation at 28 °C in SDB.

Sample preparation for electron microscopy

The samples were washed three times in a sodium cacodylate buffer solution, 0.1 M (pH 7.2), and fixed for 12 h at 4 °C in a 4% glutaraldehyde solution in the same buffer. After 2 washes in the buffer, samples were dehydrated in ethylic alcohol and dried by critical point technique (CO_2; Balzers). They were metallized with or-palladium (20 à 30 nm) in a cathodic evaporator (Jeol, JFC-100). Observations were made at 20 KVolt by scanning electron microscopy (Cambridge stereoscan S-360).

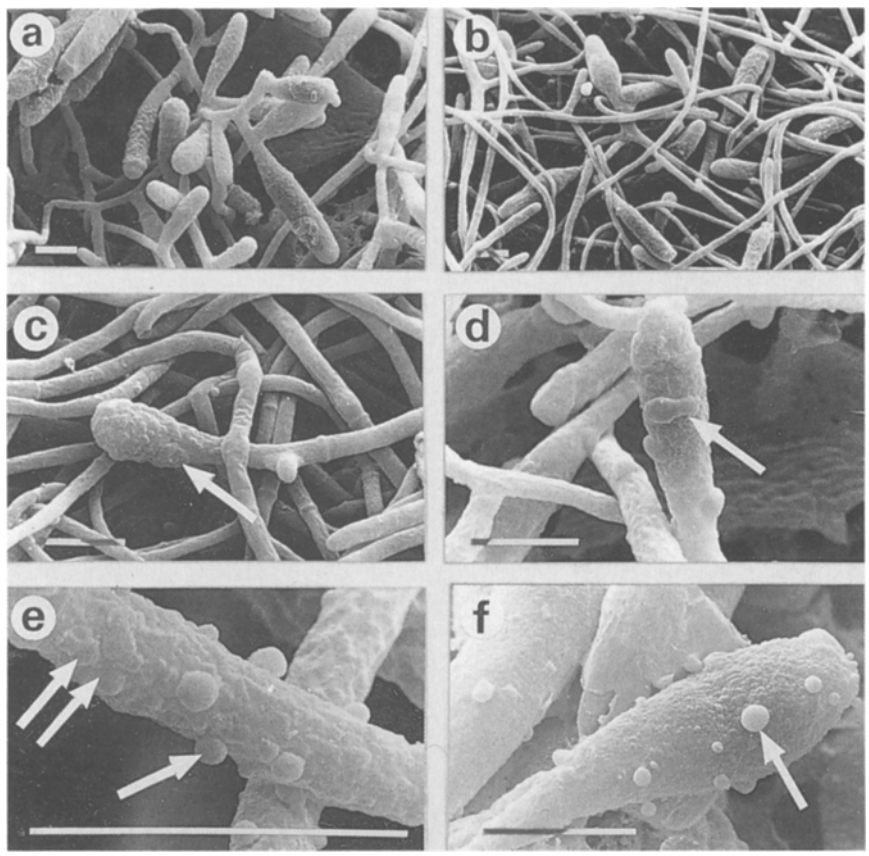

Fig. 1. Epidermophyton floccosum. (a) Non-treated sample after 7 days cultivation: The macroconidia are sometimes grouped in clusters; (b) Non-treated sample-DMSO after 7 days cultivation: The mycelial filaments are cylindrical and not very dichotomous; bludgeon-shaped macroconidia are often isolated; (c) Non-treated sample-DMSO after 7 days cultivation: Filaments are turgescent with visible septa. Macroconidia are covered with bumps (arrow); (d) Non-treated sample after 7 days cultivation: Macroconidia showing bulges (arrows); (e) Non-treated sample after 7 days cultivation: Hyphal filament showing bumps (double arrow) and vesicles (arrow); (f) Non-treated sample after 7 days cultivation: Macroconidia sparsely covered with vesicles (arrow). bar = 10 μm.

Results and discussion

Control samples of *E. floccosum* showed a classic organization of structure including perfectly conserved mycelium hyphae and bludgeon-shaped conidia isolated (Fig. 1a) or disposed-like clusters (Fig. 1b). Mycelial hyphae were normally cylindrical and smooth. We observed more rarely a few ornamentations on hyphae as well as on macroconidia. These ornamentations, already found by other authors [2] are characterized by 'warty swelling pustula' on hyphae (Fig. 1e) and on macroconidia (Fig. 1f). They are irregularly disposed and randomly distributed within the mycelium or on macroconidia. In addition to these 'pustula', macroconidia also displayed bulges (Fig. 1d) on a par with the transverse septum. Young macroconidia were more swollen (Fig. 1c) and their wall less smooth than more mature conidia. Alterations induced by the allylamines on treated samples involved both mycelial filaments and macroconidia. With a concentration equal to the MIC (0.2 $\mu g\,ml^{-1}$ for naftifine and 0.01 $\mu g\,ml^{-1}$ for terbinafine) numerous hyphae were twisted (Fig. 3f, 2b) with swellings of hyphal tips (Fig. 2b, c, 3c) and septa (Fig. 2e, f). Aborted bud-

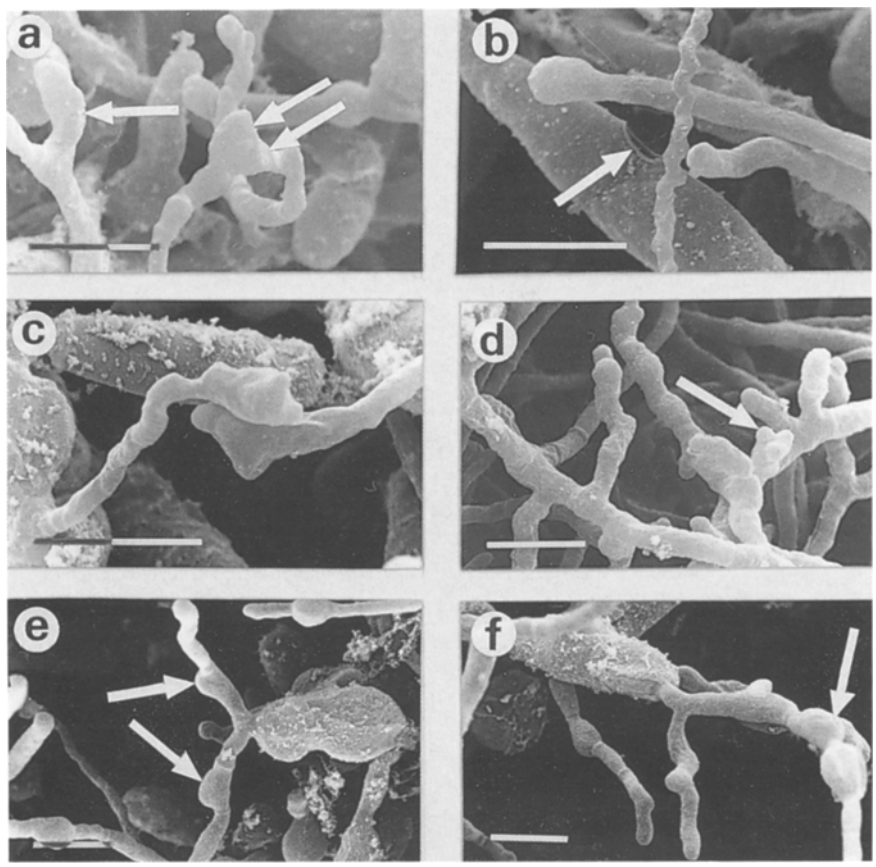

Fig. 2. Epidermophyton floccosum. (a) Sample treated for 3 days with 0.2 $\mu g\,ml^{-1}$ naftifine. Swelled lateral buds (arrow) and distorted mycelium (double arrow); (b) Sample treated for 7 days with 0.2 $\mu g\,ml^{-1}$ naftifine. Bulbous apex and twisted filament. Macroconidia are perforated (arrow); (c) Sample treated for 3 days with 2 $\mu g\,ml^{-1}$ naftifine. Mycelial hyphae are very deformed and stem from macroconidia altered by the exfoliation phenomenon; (d) Sample treated for 3 days with 2 $\mu g\,ml^{-1}$ naftifine. Notice the numerous lateral aborted and swollen ramifications (arrow); (e) Sample treated for 7 days with 2 $\mu g\,ml^{-1}$ naftifine. Swelling on hyphae near the septum (arrow); (f) Sample treated for 7 days with 2 $\mu g\,ml^{-1}$ naftifine. Abnormal bulging filament (arrow) growing from the altered macroconidia. bar = 10 μm.

dings were frequently observed (Fig. 2a, d). Meingassner et al. [3] made exactly the same observations using *Trichophyton mentagrophytes* treated by 1 $\mu g\,ml^{-1}$ of naftifine for 48 h. These deformations also were noted by Dall'olio et al. [4] on *T. mentagrophytes* under the action of coumarin and by Borgers [5] on *Aspergillus fumigatus* after exposure to saperconazole.

Macroconidia were distorted with concentrations of naftifine and terbinafine approaching the MIC (Fig. 2b, 3a, b). These structural alterations underwent important modifications when exposed to drug concentrations ten times higher than the MIC. The outer wall-layer works loose; the residues formed in this way take on a fluffy appearance, become detached (Fig. 3a, b, c) and agglomerate on the smooth layer beneath and on the hyphal filaments which are nearby (Fig. 3e). It would appear that macroconidia with old hyphae are invariably exposed to this phenomenon in the presence of naftifine and terbinafine, whereas, the younger filaments situated in the same observation plane did not seem altered in the same way (Fig. 2c–f, 3c).

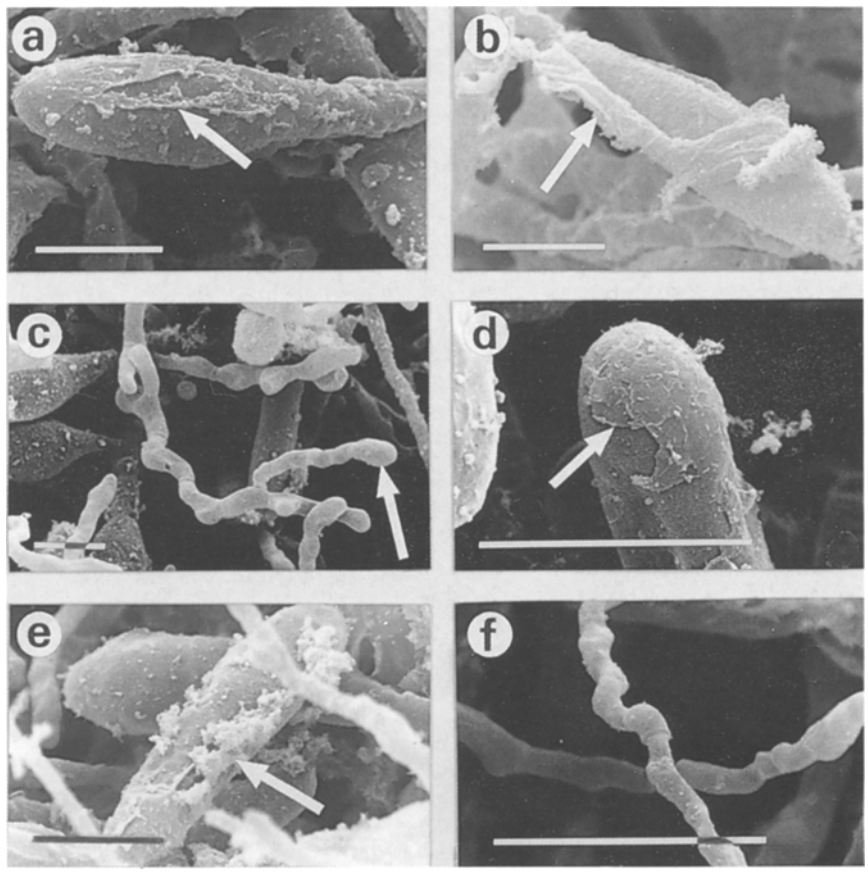

Fig. 3. Epidermophyton floccosum. (a) Sample treated for 24 h with 0.01 $\mu g\ ml^{-1}$ terbinafine. Macroconidial cell wall exfoliation (arrow); (b) Sample treated for 3 days with 0.01 $\mu g\ ml^{-1}$ terbinafine. Macroconidial cell wall in tatters (arrow); (c) Sample treated for 7 days with 0.01 $\mu g\ ml^{-1}$ terbinafine. The hyphal filaments are deformed with bulbous apex (arrow); (d) Sample treated for 3 days with 0.1 $\mu g\ ml^{-1}$ terbinafine. Distal macroconidial end with wall in the process of exfoliation (arrow); (e) Sample treated for 7 days with 0.1 $\mu g\ ml^{-1}$ terbinafine. The cell wall macroconidial residues agglomerate and disperse within the mycelium; (f) Sample treated for 7 days with 0.1 $\mu g\ ml^{-1}$ terbinafine. Twisted filaments with variable diameter. bar = 10 μm.

The macroconidia became brittle at the extremity and released their cytoplasm via a large hole that formed in the wall (Fig. 2b). These lesions could be explained by uncontrolled autolytic enzyme activation in the fungal cell which could induce their destruction. Kitazima et Nozawa [6] succeeded in isolating the constitutive external layer of the *E. floccosum* cell wall. They concluded from this that the cell wall was structured by plurilamellar layers and the most external layer has a different chemical composition from the rest of the wall. Hellgren et al. [7] noticed macroconidia perforations of *E. floccosum* under the action of n-undecanoic acid solution on a strain of this species. The exfoliation phenomenon induced by an antifungal already has been noted by Osumi et al. [8] who studied the activity of bifonazole on *T. mentagrophytes* mycelium. The intensity of these alterations is dependent on the allylamine concentrations and is noticeable very soon after the MIC is attained; considerable mycelia damage occurs at this concentration.

Allylamines inhibit the squalene epoxidase which permits the passage of squalene to expoxido squalene [9]. Epoxido squalene serves as a substrate for other enzymes such as ergosterol

biosynthesis [10]. Squalene epoxidase inhibition leads to squalene accumulation and a failure of ergosterol synthesis. The lack of ergosterol distorts the membranous fluidity and disorganizes the plasmic membrane structure. Chitinase which regulates chitin synthase activity and establishes cell wall shape and rigidity [11] as well as glycoprotein and phospholipid biosynthesis also are inhibited [12]. Lastly, the accumulation of squalene in the plasmic membrane, in correlation with an abnormal chitin deposit, induces the formation of vesicles which are released either in the inner cell, making the filament look swollen, or between the wall and the membrane making irregularly distributed swellings appear on the hyphal filament. Meingassner et al. [3] and Ryder [13] observed the formation of numerous vesicles on both sides of the cell wall, which could be caused by an accumulation of squalene.

Metabolism is accelerated in hyphal ends as demonstrated by Blank et al. [14] who affirmed that organelle density in hyphae is dependent on cellular metabolism. They found numerous mitochondrians in the hyphal ends indicative of high oxidative metabolism. Poulain et al. [15] showed that young filaments have abundant organelles with intense metabolic activity.

Naftifine and terbinafine, by inhibiting the synthesis of the cell wall and plasmic membrane, induce an accumulation of substrates at hyphal tips. Indeed, we can conceive that the complete inhibition of cellular metabolism is not immediate in contrast to that of the enzymes which are responsible for chitin and phospholipid synthesis. This time lag therefore may give rise to an overloading of the metabolite precursors of cell wall and plasmic membrane synthesis as well as to the overloading of squalene. This could explain in part the bulbous tips which we observed. The filaments therefore cannot grow lengthwise. However, they emit numerous lateral ramifications which then are aborted. Mycelia takes on a particular shape with twisted, tortuous and aborted short filaments. Thus, instead of unidirectional growth, it would seem that the allylamine-treated filament shows a trend toward multidirectional growth induced by apex growth inhibition. The causes of these deformations still remain doubtful and must be confirmed by a study using transmission electron microscope. However, this current study of morphological changes induced by allylamines on *E. floccosum* conidia and mycelia confirms that naftifine and terbinafine display excellent antifungal activity against dermatophytes.

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