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Full-length paper

Effects of micafungin on the morphology of *Aspergillus fumigatus*

Yayoi Nishiyama*, Yayoi Hasumi, Kumiko Ueda, Katsuhisa Uchida and Hideyo Yamaguchi

Teikyo University Institute of Medical Mycology, Hachioji, Tokyo 192-0395, Japan
*To whom correspondence should be addressed. E-mail: ynishiya@main.teikyo-u.ac.jp

Abstract The morphological effects of micafungin, a member of a new class of antifungal agents candins, on growing hyphae of *Aspergillus fumigatus* were studied by the use of Nomarski microscopy, scanning electron microscopy and thin-section electron microscopy. Micafungin at concentrations of 0.001–0.1 μg ml^{-1} strongly inhibited the *in vitro* growth of this fungus and induced striking changes in the hyphal morphology, depending on the drug concentration and the length of the incubation period. The changes observed included increased formation of branches on the lateral walls, disruption of the tips of both hyphal cells and branches, and crushing and collapse of whole hyphae. In addition, micafungin was also effective in damaging membranous structures, including disruption of the cell membrane, partial loss of nuclear membranes and expansion of endoplasmic reticula. From these results, it was concluded that micafungin primarily affected the normal formation of cell walls and septa of growing hyphae. The inhibition of apical growth of hyphae accompanied by excessive lateral branching that was followed by disruption of both hyphal tips and branch tips ultimately led to the destruction of whole hyphae. This cytological effect of micafungin on the hyphal growth of *A. fumigatus* can explain its potent anti-*Aspergillus* activity.

Keywords micafungin, antifungal agents, candins, *Aspergillus fumigatus*, morphology, electron microscopy

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Introduction

With the increasing use of various modern aggressive medical cares, the incidence of serious invasive fungal infections, particularly those caused by *Candida* spp. and *Aspergillus* spp., has continued to increase in immunologically or physiologically compromised patients [1–3]. However, antifungal agents approved for the treatment of invasive fungal infections are limited in number and clinical usefulness, and fungal isolates resistant to the available drugs have become more prevalent [4,5]. Therefore, the development of new antifungal agents with increased clinical usefulness in terms of efficacy and/or safety is needed.

Extensive research and developmental studies have been carried out on a new class of antifungal agents, named candins, which characteristically possess a unique structure composed of a cyclic hexapeptide linked with a hydrophobic long-chain acyl residue. Several candins have been demonstrated to exhibit selective and non-competitive inhibition of the 1,3-β-D-glucan synthase that catalyses the synthesis of 1,3-β-D-glucan, an essential component of the fungal cell wall, eventually leading to blockage of normal cell wall formation [6–11]. Currently, two candins, caspofungin and micafungin, respectively, have been introduced for clinical use in the US and Japan.

Micafungin is a water-soluble, parenterally administered antifungal drug that has a potent *in vitro* and *in vivo* activity against many clinically important fungi, especially *Candida* spp., including azole-resistant isolates, and *Aspergillus* spp. [12– 16]. Like other candins, micafungin inhibits the synthesis of 1,3-β-D-glucan in susceptible yeasts and filamentous fungi [17].

Morphological approaches are considered particularly important to learn the outcome of the action of cell wall-active antifungal agents, such as micafungin. The effect of micafungin on the morphology or ultrastructure of *C. albicans* has been studied by Nishiyama *et al*. [18], but there are no existing similar reports describing the alterations produced by this drug on *A. fumigatus*, with the exception of a study by Chiou *et al*. [19], which dealt mainly with the morphological changes of the filamentous fungus produced by nikkomycin Z and micafungin used in combination.

The present paper describes the effects of micafungin on the gross morphology as well as on the ultrastructure of the cell surface and the cell interior of growing hyphae of *A. fumigatus* that were observed by Nomarski microscopy, scanning electron microscopy (SEM) and thin-section electron microscopy (TEM).

Methods

Antifungal drug

Micafungin was obtained from Fujisawa Pharmaceutical Co., Ltd (Osaka, Japan). Stock solutions of the drug were prepared at concentrations of 1–0.1 mg ml^{-1} in physiological saline.

Organism

Aspergillus fumigatus TIMM 3968, a stock strain originally isolated from a patient with pulmonary aspergillosis, was used in this study.

Culture conditions and treatment with micafungin

The organism was grown on Sabouraud dextrose agar slants at 30°C for 1–2 weeks until conidia were fully mature. Conidia were collected by rubbing the surface of the slants with a metal loop after addition of sterile phosphate-buffered saline (PBS), pH 7.2, containing 0.01% Tween-80. Hyphal debris was removed by filtration through sterile cotton gauze and by centrifugation, and conidia yielded were suspended in PBS. The final concentration of the conidial suspension was adjusted to 2×10^8 conidia ml^{-1} using a standard haemocytometer and diluted to $2–5 \times 10^5$ conidia ml^{-1} in RPMI 1640 medium (buffered to a pH of 7.0 with 0.165 M morpholinepropanesulphonic acid; both from Sigma Chemical Co.) to give the experimental conidial suspension.

To prepare growing hyphal cultures, 900-µl aliquots of the experimental conidial suspension were dispensed into each well of Falcon 24-well flat-bottom plates (Becton Dickinson and Company, NJ, USA). When most conidia had begun to germinate, 100-µl aliquots of micafungin solution of various drug concentrations were added to each well and incubated at 30°C without shaking. After 2 and 5 h of incubation, cultures were harvested by centrifugation and processed for Nomarski and electron microscopy.

Determination of the MIC

The minimum inhibitory concentration (MIC) of micafungin for the *A. fumigatus* strain was obtained by the broth microdilution method following the Japanese Society for Medical Mycology (JSMM) technique for filamentous fungi using RPMI 1640 medium supplemented with a redox indicator (Alamar Blue™; Alamar Bioscience Inc.) as the test medium, an inoculum of 1.2×10^4 conidia ml^{-1} and an incubation temperature of 30°C [20]. The end-point was determined visually as the lowest concentration at which the blue colour just turned reddish or by spectrophotometric measurement using a microplate reader at 570 nm, as the lowest concentration showing $\leq$20% of the absorbance of the growth control (calculated IC_{80}), as described before [12].

Nomarski microscopy

Cultures were rinsed with 0.1 M cacodylate buffer (pH 7.2) and placed on microscopic slides. They were examined with Nomarski differential interference contrast on a Leica DMR-HC microscope (Leica Microsystems, Germany), as described previously [18].

Electron microscopy

For SEM, cultures were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4°C for 2 h. After being washed with the buffer, specimens were post-fixed for 2 h with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) at 4°C. Samples were dehydrated in graded acetone, freeze-dried in *t*-butyl alcohol and sputter-coated with palladium–gold as described previously [21]. Observations were carried out with a JEOL 5400 LV scanning electron microscope.

For TEM, cultures fixed with 2% glutaraldehyde solution as described above were post-fixed for 18 h with 1.5% potassium permanganate at 4°C. After three wash steps in water, specimens were dehydrated in a series of graded acetone and embedded in Quetol 653. Thin sections were stained with uranyl acetate followed by lead citrate and examined in a Hitachi H-7000 electron microscope operated at 75 KV, as mentioned in our previous papers [18,21].

Results

Susceptibility testing

In the present study, although the susceptibility testing method for micafungin has not been standardized yet, the JSMM method for filamentous fungi was used and IC_{80} as the spectrophotometric end-point adopted to determine the MIC for micafungin. The MIC of micafungin thus obtained for *A. fumigatus* TIMM 3968 was $\leq$0.0078 µg ml^{-1}.

Effect of micafungin on the hyphal growth of *A. fumigatus*

Under the present experimental conditions, marked reduction in the turbidity of the growing cultures was seen when

treated with 0.001 μg ml^{-1} micafungin, the concentration almost corresponding to one-tenth of its MIC. In the following morphological studies, three different drug concentrations were used to treat cultures: one-tenth of the MIC (0.001 μg ml^{-1}), the MIC (0.01 μg ml^{-1}) and 10 times the MIC (0.1 μg ml^{-1}) of micafungin.

Nomarski microscopic studies

Nomarski microscopy was initially used to observe gross morphology of cultures treated with or without micafungin. In control cultures grown without micafungin for 2 or 5 h, hyphae elongated normally with dichotomous branching in a Y-shape (Figs 1a and 1b). In contrast, micafungin treatment caused marked alterations of the hyphal morphology, depending on the drug concentration and the length of the treatment period. When treated with 0.001 μg ml^{-1} micafungin for 2 h, numerous sprouts of branches were observed on the lateral walls of the hyphae with occasional collapse of hyphae and the inter-septal distance of the hyphae appeared shorter than that for untreated control cultures (Fig. 1c). After 5 h of treatment with 0.001 μg ml^{-1} micafungin, most of the branched tips and hyphae elongated only slightly to form truncated shapes (Fig. 1d). Virtually the same morphological changes were also seen in cultures treated with higher concentrations of micafungin (0.01 and 0.1 μg ml^{-1}), and such morphological changes increased in extent with increasing drug concentrations and length of treatment period, ultimately resulting in disruption of hyphal tips or collapse of whole hyphae (Figs 1e–h).

Electron microscopic studies

Figures 2 and 3 show SEM and TEM images of untreated control cultures grown for 2 h, respectively. A SEM micrograph demonstrates that hyphae of *A. fumigatus* with the smooth surface were uniform in width (1.5–2 μm) and straightly elongated with few branches. The residue of germinated conidia with granular protrusions on the surface attached to the basal portion of the hyphae was seen occasionally (Fig. 2, arrow). Figure 3 shows a TEM image of a hyphal tip in control cultures typically representing cytoplasmic organelles, such as mitochondria and endoplasmic reticula as well as the nucleus. The cell membrane appeared as a sharp, electron-dense lipid bilayer closely attached to the inner face of the fungal cell wall. In the apical region of the hypha, small vesicles (Spitzenkörper) ranging from 50 to 100 nm in diameter dominated the cytoplasm (Fig. 3, arrow). The cell wall at the tip of the hypha was even in thickness (80–100 nm), and comprised an outer fibrillar layer displaying a sparsely distributed, coarse, electron-dense layer on the cell wall surface and an inner layer appearing as a uniform, electron-transparent region just external to the cell membrane. This wall structure pattern did not vary through the transition to the subapical region. Virtually the same ultrastructural feature of hyphal tips was seen in control cultures grown for 5 h (data not shown).

In contrast, when micafungin-treated cultures were observed by SEM, three prominent types of change in hyphal morphology were produced: (i) formation of numerous short branches on the lateral walls; (ii) distortion and collapse of whole hyphae; and (iii) disruption of tips of both hyphae and lateral branches. Although such types of hyphal change were similarly seen in all micafungin-treated cultures, whatever the concentration (0.001, 0.01 and 0.1 μg ml^{-1}), the changes became more intense and frequent with increasing drug concentration and length of treatment time.

Figure 4 illustrates a SEM image of a typical morphological feature of lateral branches of hyphae after treatment with 0.1 μg ml^{-1} micafungin for 2 h. Highly branched hyphae were characterized by swollen tips with rough surfaces (Fig. 4, arrow). Furthermore, some hyphae showed distortion, collapse and/or shrinkage (Fig. 4). Similar changes in the hyphal morphology, although produced less frequently, were also seen in the culture treated with micafungin at concentrations of 0.01 or 0.001 μg ml^{-1} for 2 h (data not shown). Although hyphae were less severely damaged when cultures were treated with 0.01 μg ml^{-1} of micafungin for 5 h, almost all hyphae were collapsed with some disrupted hyphal tips, from which intracellular materials were released externally (Fig. 5). As shown in Fig. 6, after treatment with 0.1 μg ml^{-1} micafungin for 5 h, more severe changes, including almost complete collapse and crush of hyphae, were produced. On the surfaces of such disrupted hyphae, irregularly shaped materials that seemed to be intracellular materials leaked externally were seen (Fig. 6, arrow).

Ultrastructural modifications of the cell wall and cell interior of micafungin-treated hyphae were revealed by TEM. Major changes are the disorder of septum formation, and accumulation of electron-dense inclusions in the cell wall and in the space between the cell membrane and the cell wall. Figure 7 shows a typical feature of hyphae with incompletely or aberrantly formed septa, leading to disproportionate segmentation of hyphae, which were seen in cultures treated with 0.1 μg ml^{-1} micafungin for 5 h.

Figure 8 shows the ultrastructural details of the tip of a lateral branch of hyphae after treatment with 0.001 μg ml^{-1} micafungin for 5 h. At the tip of the branch, many electron-dense inclusions, varying in size and shape, were observed within the cell wall and the space between the wall and the cell membrane, although no remarkable ultrastructural change was found in the cytoplasm of the branch (Fig. 8). A TEM image of disrupted hyphal tips, one of the most noticeable micafungin-induced morphological changes is illustrated in Fig. 9. Most parts of the cell wall and cell membrane of the apical cell were lost (or hardly observed) after 2 h treatment with 0.01 μg ml^{-1} micafungin (Fig. 9).

In addition to these rather drastic morphological or ultrastructural changes, impairment of membrane structures in the cytoplasmic organelles and nucleus was observed occasionally in relatively intact hypha in micafungin-treated cultures. As typically shown in Fig. 10, disruption of cell membranes, partial loss of the nuclear membrane and

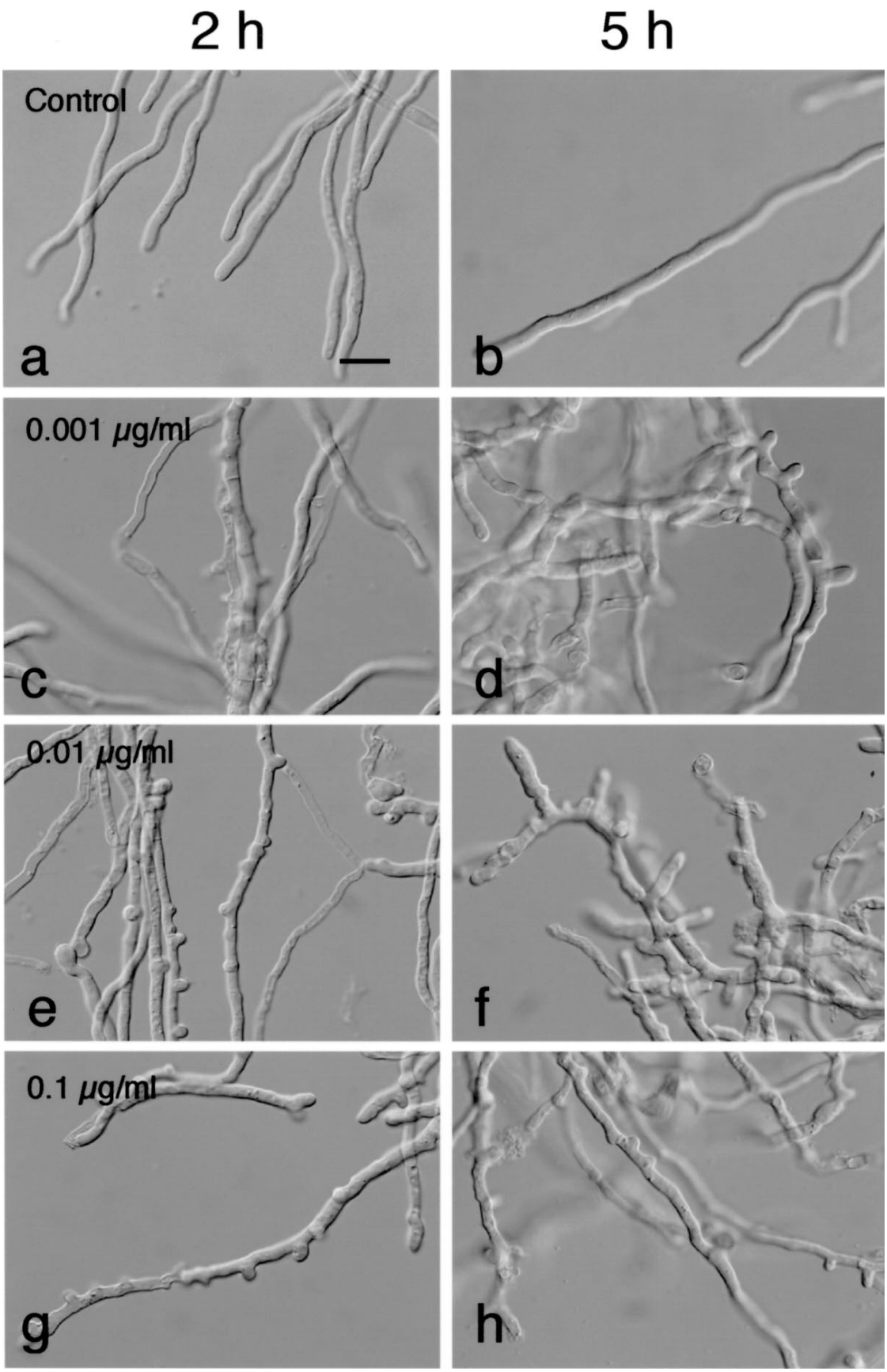

Fig. 1 Nomarski micrographs of *A. fumigatus* cultures grown for 2 h (a) and 5 h (b) in drug-free RPMI 1640 medium, and those in medium supplemented with micafungin at 0.001 μg ml^{-1} (c and d), 0.01 μg ml^{-1} (e and f) and 0.1 μg ml^{-1} (g and h), respectively. Bar = 10 μm.

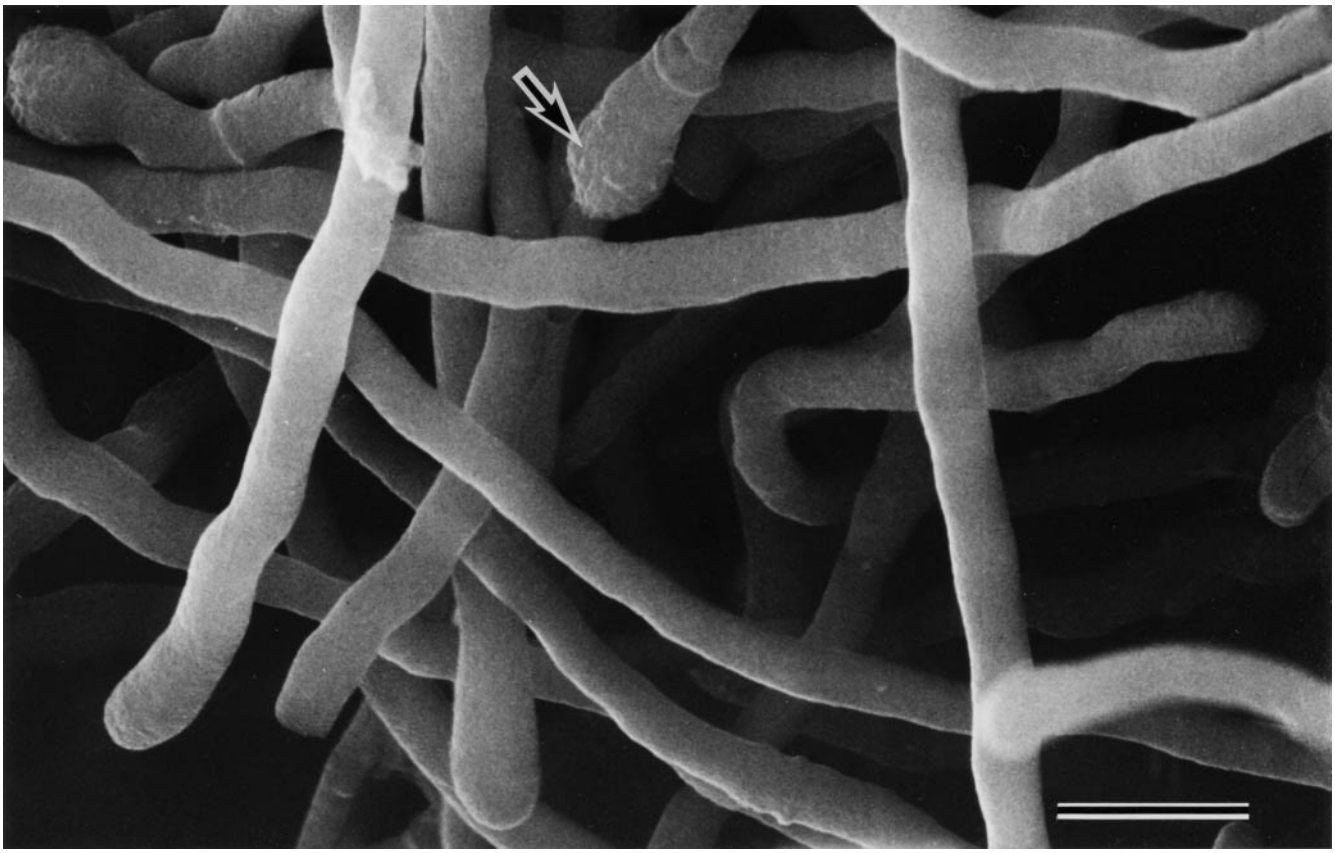

Fig. 2 SEM image of untreated control hyphae of *A. fumigatus* grown for 2 h, showing straight elongation of hyphae with smooth surfaces. Germinated conidia (arrow) are seen in the basal portion of a hypha. Bar = 5 μm.

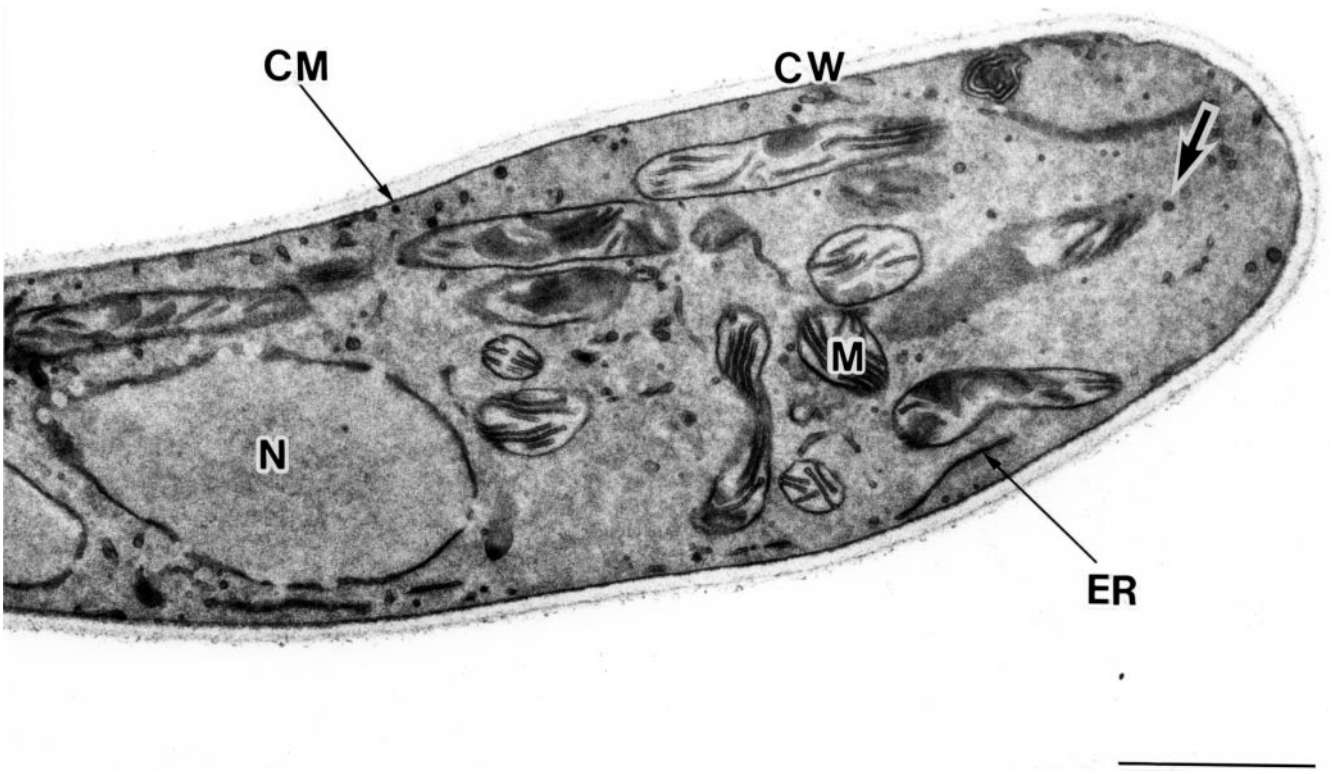

Fig. 3 TEM image of untreated control hyphal cell grown for 2 h. Abbreviations: N, nucleus; M, mitochondria; ER, endoplasmic reticulum; CM, cell membrane; CW, cell wall. The arrow indicates a Spitzenkörper. Bar = 1 μm.

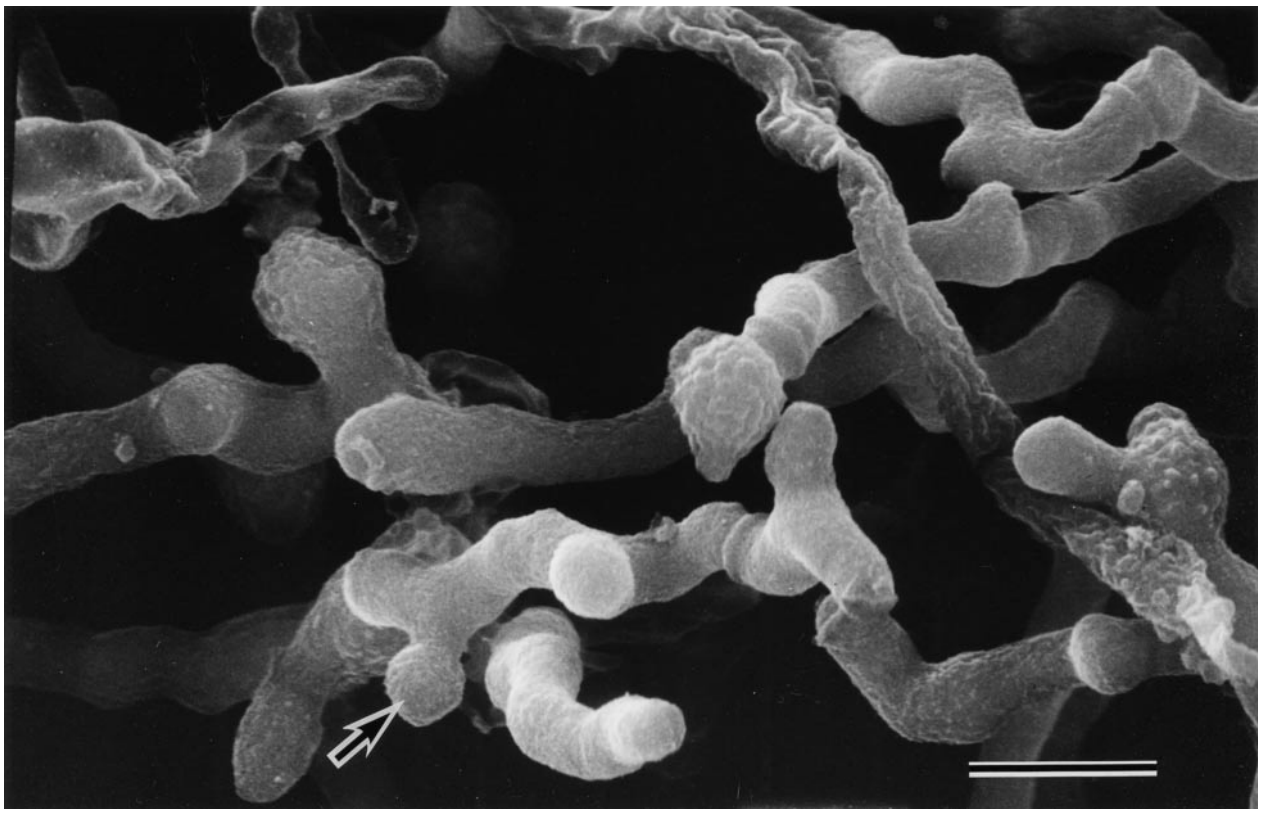

Fig. 4 SEM image of hyphae grown for 2 h with 0.1 μg ml^{-1} micafungin, showing short branch with a rough and wrinkled surface. Note the swollen tips (arrow) on the short branch. Bar = 5 μm.

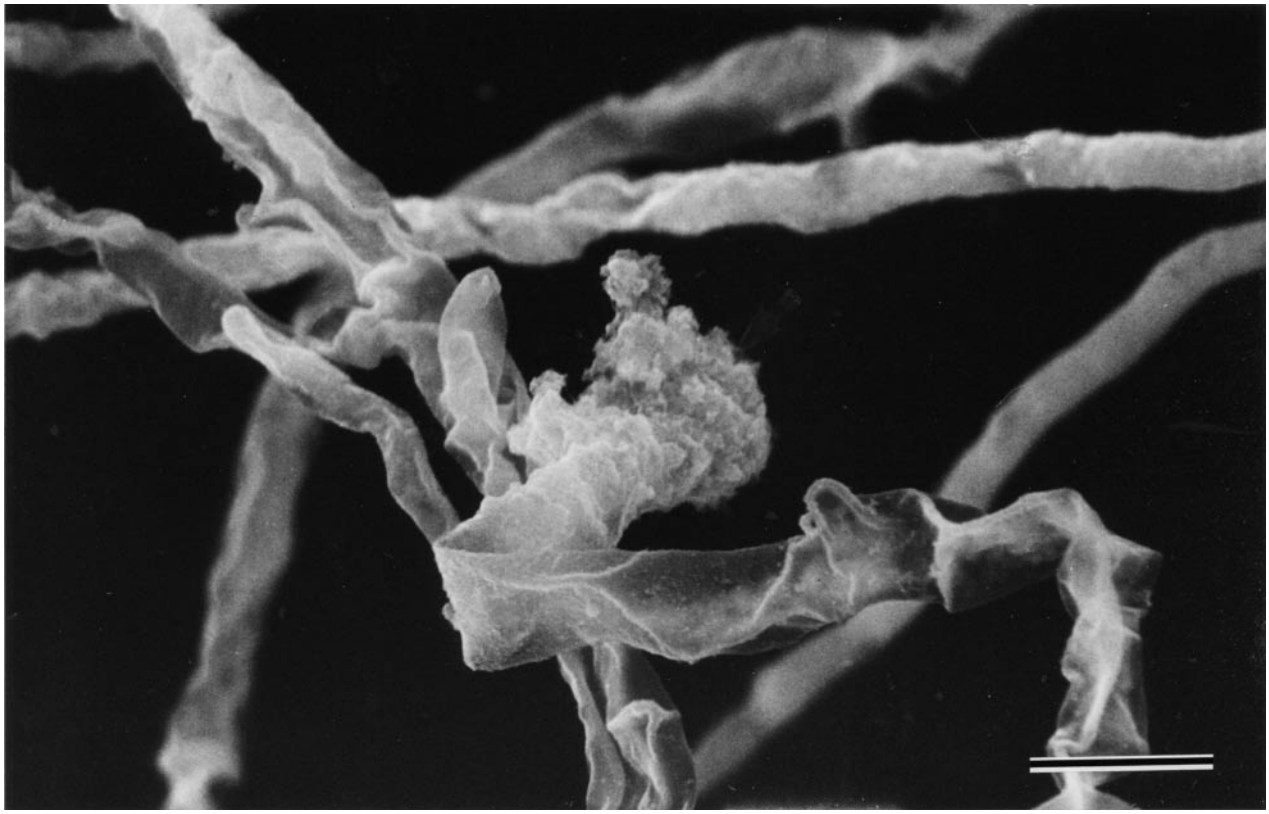

Fig. 5 SEM image of hyphae grown for 5 h with 0.01 μg ml^{-1} of micafungin, showing disruption of hyphal tips and collapse of hyphae. Bar = 5 μm.

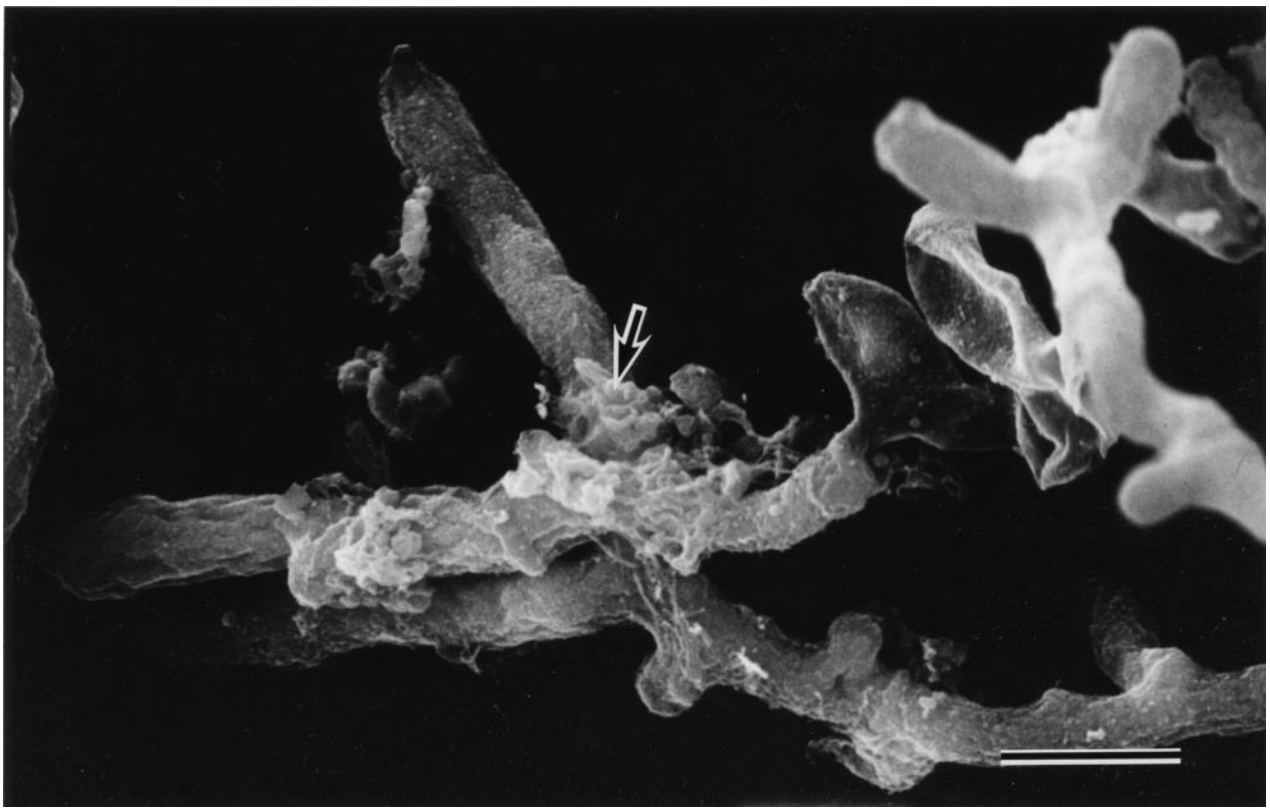

Fig. 6 SEM image of hyphae grown for 5 h with 0.1 μg ml^{-1} micafungin, showing crush and distortion of hyphal cells. Irregularly shaped materials were seen on the cell surfaces (arrows). Bar = 5 μm.

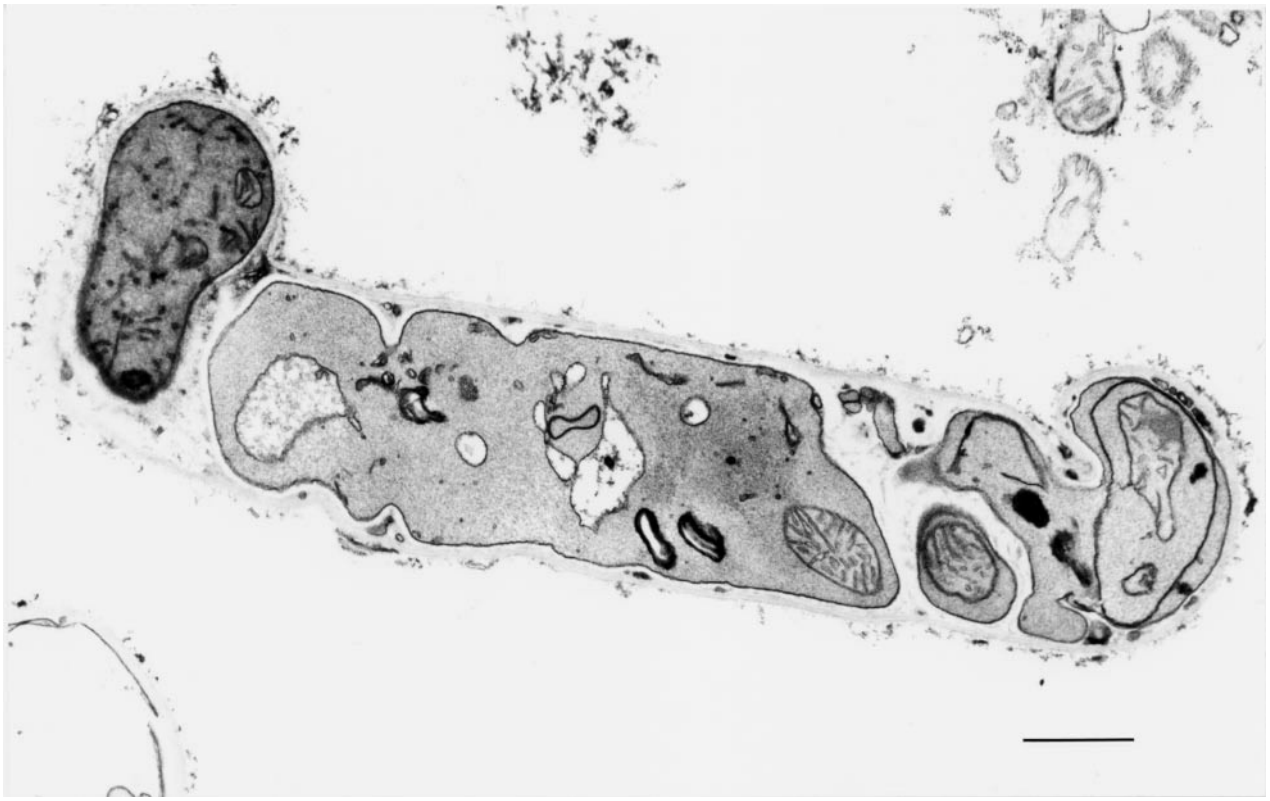

Fig. 7 TEM image of a hyphal cell grown for 5 h with 0.1 μg ml^{-1} micafungin showing abnormal formation of the septal walls. The hypha is segmented disproportionately by irregularly induced septa. Bar = 1 μm.

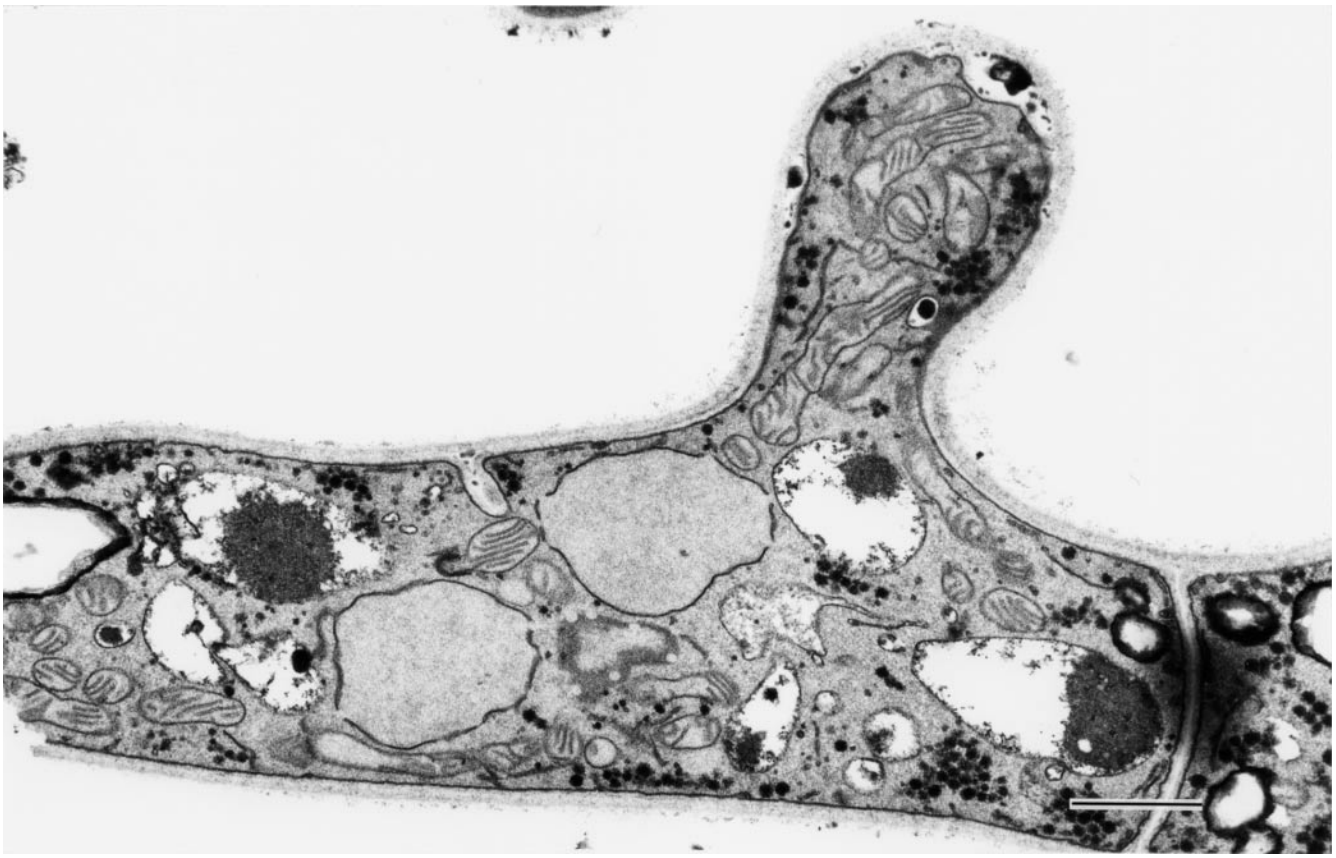

Fig. 8 TEM image of a hyphal cell grown with 0.001 μg ml^{-1} micafungin for 5 h. Note short lateral branch and electron-dense inclusions located at the tip of the branch. Bar = 1 μm.

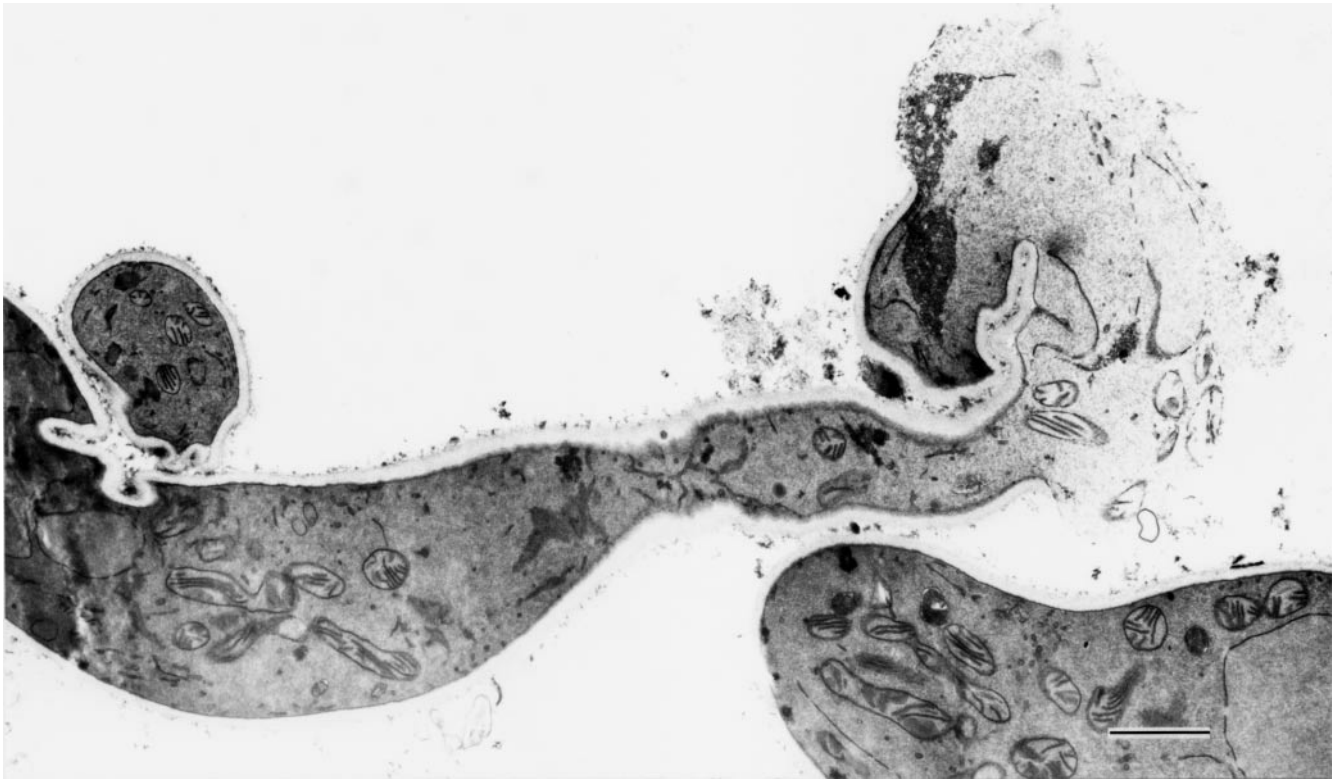

Fig. 9 TEM image of a hyphal cell treated with 0.01 μg ml^{-1} micafungin for 2 h, showing disrupted hyphal tip. Bar = 1 μm.

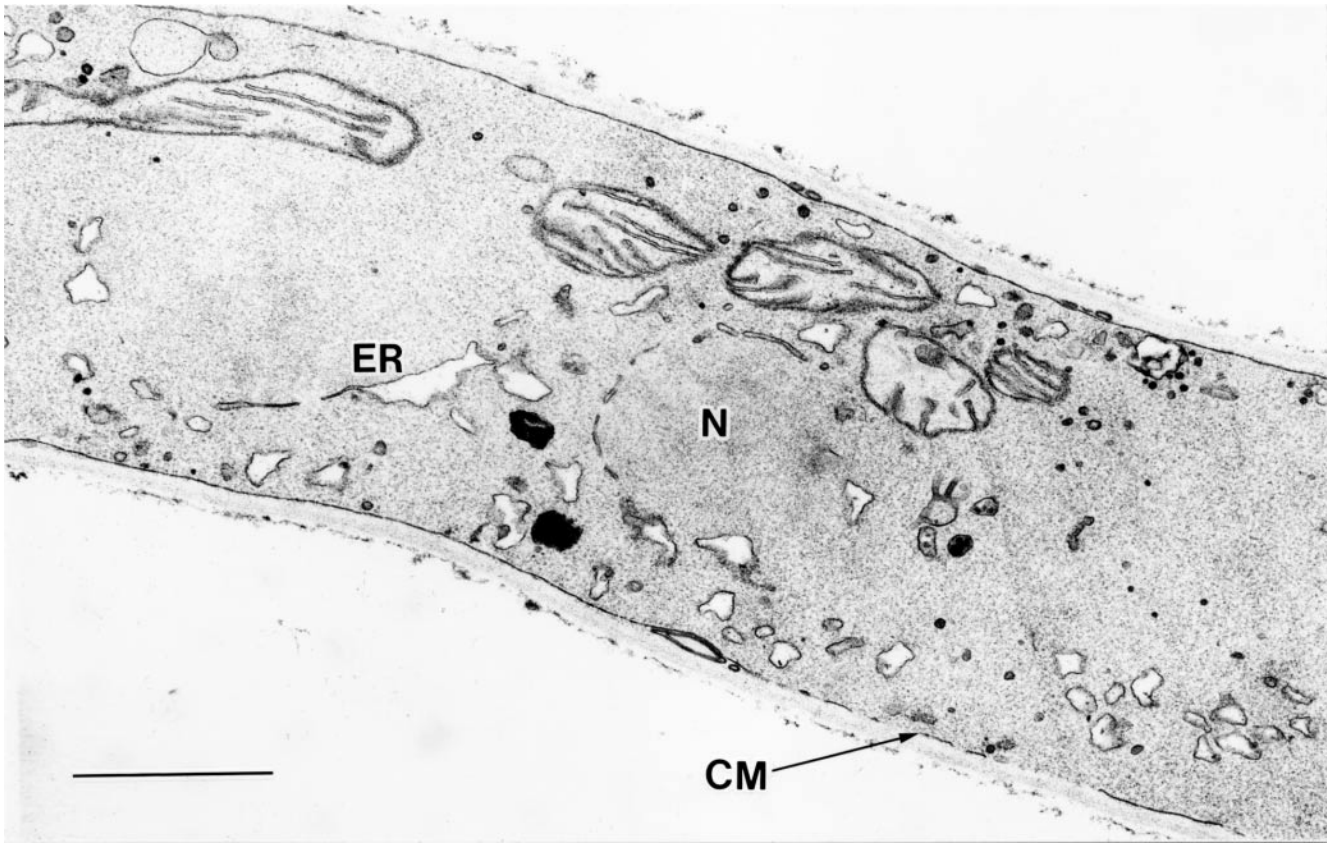

Fig. 10 TEM image of a hyphal cell treated with 0.001 μg ml^{-1} micafungin for 5 h. Note the disruption of the cell membranes, loss of portion of the nuclear membrane and expansion of the endoplasmic reticulum membrane. Bar = 1 μm.

expansion of the endoplasmic reticulum are seen in hyphae from cultures exposed to 0.001 μg ml^{-1} micafungin for 5 h (Fig. 10).

Discussion

In this study, we have demonstrated morphological and ultrastructural changes of growing hyphae as well as of constituting hyphal cells of *A. fumigatus* affected by a cell wall-active antifungal agent, micafungin, under varying treatment conditions. As in many other fungi, *A. fumigatus* contains 1,3-β-D-glucan in the cell wall as the major skeletal component [22,23]. Therefore, it is the rational speculation that inhibition by micafungin of fungal 1,3-β-D-glucan synthesis can induce abortive cell-wall formation in susceptible fungi. Actually, the primary morphological changes produced by growth-inhibitory or -subinhibitory concentrations of micafungin are those associated with the inhibition of normal cell wall formation. One of the most prominent changes was the development of numerous short branches on the lateral walls of hyphae. Although the exact reason remains to be answered, this abnormal hyphal growth might be a fungal response that compensates the inhibited apical growth of hyphae. Similar morphological changes were reported previously by Chiou *et al.* [19] and Kurtz *et al.* [24], who studied micafungin and pneumocandins, respectively.

Apical growth at the hyphal tip, where hyphal metabolic activity is at its highest, is characteristic of filamentous fungi, including *A. fumigatus* [25,26]. Although biochemical and molecular biological studies on the growth of filamentous fungi lag far behind those on the growth of yeasts, the localization of 1,3-β-D-glucan synthase was reported recently to be at hyphal tips [27]. Thus, it is not surprising that the hyphal tip, as well as the lateral branch tip, is most susceptible to cell wall formation-inhibitory activity of micafungin, eventually resulting in complete or almost complete disruption of this part of the hyphae during 5 h of incubation. The disruption of tips of both hyphae and lateral branches may induce extracellular leakage of cellular materials, ultimately leading to the collapse or autolytic destruction of whole hyphae. These morphological effects of relatively low concentrations of micafungin could explain its potent fungicidal action towards growing cultures of *A. fumigatus* and other susceptible filamentous fungi.

In addition to the effect on cell wall formation, micafungin was also effective in injuring membranous structures, i.e. partial loss of the nuclear membrane and expansion of the endoplasmic reticulum membrane in hyphal cells of *A. fumigatus*. Similar morphological changes have been

observed in *C. albicans* cells treated with micafungin [18] and related lipopeptide compounds [28–30].

In the TEM image of micafungin-treated hyphae of *A. fumigatus*, deposition of electron-dense vesicles and/or irregularly shaped membranous materials was observed within the cell walls or septa. Comparable deposits were also found in the cell walls of hyphal cells of *Trichophyton mentagrophytes* and *C. albicans* treated with inhibitors of ergosterol synthesis, such as imidazoles, allylamine and morpholine, which are known to damage secondarily the cell membrane, because ergosterol is an essential lipid component of the cell membrane [31–34]. These results lead us to the possibility that micafungin is active against the fungal cell membrane, probably due to its amphiphilic physicochemical nature.

The morphological effect of micafungin on hyphae of *A. fumigatus* appears to be different from that on yeast cells of *C. albicans*, in which the drug principally inhibited normal formation of both peripheral and septal walls, typically represented by thinning of the peripheral walls and irregular-shaped septum formation, resulting in swollen and osmotically fragile yeast cells and abortive budding [18]. However, this may reflect differences in the manner of growth between a filamentous fungus and budding yeast.

Concluding remarks

We examined the effects of micafungin, a member of a new class of antifungal agents, candins, on the morphology of growing hyphae of *A. fumigatus* by Nomarski and electron microscopic techniques. Hyphal growth of cultures was inhibited and aberrant morphology of hyphae induced when incubated with 0.001–0.1 μg ml^{-1} micafungin at 30° C for 2 or 5 h. Characteristic morphological changes of hyphae were frequent formation of branches on hyphal lateral walls, disruption of the tips of both hyphae and branches, and collapse or crushing of the whole hyphae. These morphological changes could explain a potent antifungal activity of micafungin against *A. fumigatus*.

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