*Medical Mycology* 1998, **36**, 379–385 Accepted 10 February 1998

# Morphological study of *Trichophyton rubrum*: ultrastructural findings after treatment with 4-amino-3-methyl-1-phenylpyrazolo-(3,4-*c*)isothiazole

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The antifungal activity of 4-amino-3-methyl-1-phenylpyrazolo-(3,4-*c*)isothiazole was studied on *Trichophyton rubrum*. The compound, at concentrations between 20 and 100 $\mu$g ml$^{-1}$, induces a remarkable reduction in the growth and causes deep morphogenetic anomalies. The ultrastructural modifications have demonstrated that the compound targets the cell membrane of the fungus, breaking down not only the endomembrane system, but also the 'outer' membrane, with consequent extrusion of materials in the medium. The results suggests a mechanism of action similar to other azoles clinically utilized.

**Keywords** azole derivative, *Trichophyton rubrum*, ultrastructural findings

## Introduction

Despite its ever-increasing epidemiological and clinical importance and its nearly universal distribution [1], the morphology of *Trichophyton rubrum* has been little studied. It is a strictly anthropophylic dermatophyte [2], a cosmopolitan species stemming from contact through human migration. Although it is found throughout the world, it has a predilection for certain circumscribed areas (i.e. the non-woolly sporigen strains predominate in the Pacific islands and in southern Asia). Moreover, its presence is increasing everywhere, replacing other important species such as *T. mentagrophytes*.

Similar to all anthropophylic dermatophytes, it has an affinity for special parts of the body causing infections named according to the anatomic part involved. Tinea corporis, caused by *T. rubrum*, is an infection of the body which spreads over the chest, shoulders and upper limbs; its clinical manifestation runs from light forms to more serious forms with ring-shaped lesions or scaly spots clearly outlined by raised erythematous edges. Tinea cruris, which can be caused by both *T. rubrum* and *Epidermophyton floccosum*, involves the groin, perianal and perineal areas as well as the upper portion of the thighs. It presents brown or bronze-coloured erythematous lesions with thin, asymmetrical scales extending bilaterally along the inner thighs. Tinea manum manifests itself with widespread, unilateral hyperkeratosis of the palms and interdigital spaces, accentuated in the articular folds.

Tinea pedis, also caused by *T. mentagrophytes* and *E. floccosum*, affects the feet. There are many clinical manifestations of this disorder: the interdigitale form gives rise to maceration, flaking and cracking of the skin, particularly between the 4th and 5th toes; in the chronic hyperkeratosic form the reddened skin of the sole, heel and sides of the foot is covered with thin silvery flakes; the least serious form of inflammation is characterized by blisters, pimples and boils on the soles of the feet.

The two forms, distal or proximal, of tinea unguium result from the invasion of *T. rubrum* under the nails.

Although the perfect stage of *T. rubrum* has yet to be described, nevertheless, in analogy with other species in the genus *Trichophyton*, this fungus is systematically placed as follows: Family Arthrodermataceae, Order Onygenales, Class Ascomycetes, Phylum Eumycota.

The typical appearance of the colony cultivated in the laboratory is soft and woolly; initially it is white

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and slowly develops deep red veining on the lower surface as the culture matures. However, it is a highly variable species and can take on curious shapes: folded, at times convoluted and hairless. With regard to pigmentation, strains which are only lightly coloured – tending toward yellow or even white – are frequently encountered, making it difficult to distinguish them readily from other non-pigmented species of dermatophytes or from the same pleomorphic species (i.e. when the fungus is naturally pigment-free with a low conidial production). The saprophytic conidia form on the hyphae and exhibit several shapes: the single-cell microconidia are generally numerous and peg-shaped; the macroconidia with up to seven septa are one- to eight-celled. It is typical to find microconidia produced directly on macroconidia. When the fungus lives in a less than optimal environment, resistant forms called chlamydospores arise; these are large globular cells inserting with normal hyphae. In infected tissues, *T. rubrum* produces parasitic conidia (arthroconidia).

Because of its clinical importance and since little is known about its morphology, an ultrastructural study of this fungus was undertaken and was used as a reference test in an attempt to determine the action mechanism of a new synthetic azole, 4-amino-3-methyl-1-phenylpyrazolo-(3,4-*c*)isothiazole, code-named G8, which had previously proved its ability to inhibit the growth of 13 strains of dermatophytes [3] as well as some phytopathogenic fungi [4].

## Materials and methods

### Chemicals

4-amino-3-methyl-1-phenylpyrazolo-(3,4-*c*)isothiazole, G8, was kindly supplied by P. Giori and C. B. Vicentini (Department of Pharmaceutical Sciences, University of Ferrara, Italy), and prepared as described by Vicentini *et al*. [4]. Because it is not particularly soluble in water, the compound was dissolved in dimethyl sulphoxide (DMSO) and equivalent concentrations of pure DMSO were added to controls (maximum concentration, 0·1%). The final concentrations used in the experiments were 20, 50 and 100 $\mu$g ml$^{-1}$.

### Culture technique and treatment conditions

*T. rubrum* strain no. 4321, purchased from the Institute of Hygiene and Epidemiology Mycology (IHME), Belgium, was maintained at 4 °C as agar slants on malt agar (Difco). For the experiments, inocula were made from a culture prepared on a Sabouraud glucose agar medium (SGA) (Difco); agar plugs, 7 mm in diameter, were taken aseptically from the edge of a 10–15-day-old culture and placed on a G8-free medium until they reached their mid-log phase; a thin cellophane film was set between the fungus inocula and the culture medium. The Petri plates were incubated at $26 \pm 2$ °C in the dark for 3–5 days. Subsequently the cellophane films, with the mycelium, were aseptically transferred to plates with a medium containing the drug diluted to final concentrations of 20, 50 and 100 $\mu$g ml$^{-1}$. From this moment on, the growth rate was determined by measuring colony diameter (in millimetres) daily for 8 days. All determinations were made in triplicate in all experiments.

To verify actual fungal death, mycelium which had been kept in contact with SGA + 200 $\mu$g ml$^{-1}$ of G8 for 8 days were transferred to Petri dishes which did not contain the compound.

### Transmission and scanning electron microscopy

Three different samples of *T. rubrum* – controls and samples harvested at 24 and 96 h after the treatment with 50 $\mu$g ml$^{-1}$ – were chosen for observations by transmission (TEM) and scanning electron microscopy (SEM).

Two zones were taken into consideration: (i) apical mycelial cells (1–2 mm edges); and (ii) the innermost part containing older mycelium. These parts were processed routinely as described previously in works performed with other dermatophytes: the fungi were fixed with 6% glutaraldehyde (GA) in an 0·1 M sodium cacodilate buffer, pH 6·8 for 3 h at 4 °C, washed with the buffer solution and postfixed overnight with 1% osmium tetroxide ($OsO_4$) in the same buffer [5], or alternatively the fungi were fixed with 6% GA in an 0·1 M phosphate buffer, pH 7 for 3 h and postfixed with potassium permanganate ($KMnO_4$) 2% for 2 h [6]. The samples were subsequently dehydrated in a graded series of ethanol and embedded in Epon-Araldite resin.

As the normal GA-$OsO_4$ protocol proved inadequate for *T. rubrum*, after numerous attempts the following modifications were made to the fixation procedure: (i) prefixation with GA was extended from 3 h to 15 h at 4 °C; (ii) a 0·22 M sucrose was added to the buffer; and (iii) the residence time in ethanol 70% was extended from 15 min to overnight with the addition of 0·5% uranyl acetate.

Sections of the samples were cut with an LKB Ultratome III, stained with uranyl acetate and lead citrate, and observed with a Hitachi H 800 electron microscope at 100 kV.

For SEM, hyphae of control and treated mycelium were fixed in 6% GA in phosphate buffer, briefly postfixed in 1% $OsO_4$, rapidly dehydrated in acetone, critical

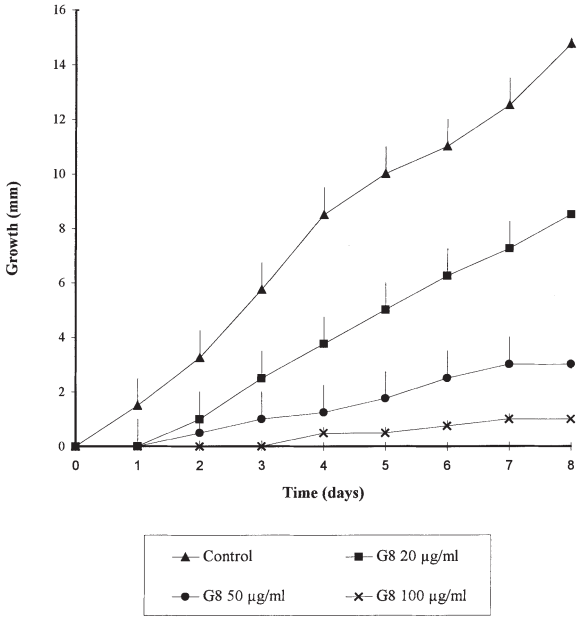



**Fig. 1** Growth curves over 8 days for *Trichophyton rubrum*. The figure shows controls, and those treated with various doses of G8.

point-dried and gold-coated in Sputter-coater S 150 (Edwards, UK). Observations were made with a Siemens Autoscan scanning electron microscope at an accelerating voltage of 20 kV.

## Results

At all the doses used 4-amino-3-methyl-1-phenyl-pyrazolo-(3,4-*c*)isothiazole significantly inhibited the growth of *T. rubrum*. This dose-dependent inhibition was 42·38, 79·67 and 93·23% at concentrations of 20, 50 and 100 $\mu$g ml$^{-1}$, respectively. Figure 1 presents the growth curves for the controls and for the fungi treated with the various doses over the 8 days of the experiment.

Given that growth was nearly totally inhibited at a dose of 100 $\mu$g ml$^{-1}$, it was decided to determine whether a drastic increase to 200 $\mu$g ml$^{-1}$ could prove lethal for the fungus. After 8 days of contact with the substance there had been no mycelium growth. Nevertheless, when the fungus was returned to a G8-free medium, growth began anew, thus indicating that even a 200 $\mu$g ml$^{-1}$ dose was solely fungistatic but not lethal. The fact that the substance was not agreeable to *T. rubrum* could be observed macroscopically. Indeed, the mycelium tended to rise up away from the G8-containing medium, indicating that the fungus could not tolerate contact.

SEM observation of the outermost, and thus youngest, portions of the control *T. rubrum* culture, grown for 24 h on SGA alone, showed mycelium made up of long strands of hyphae, normally elongated with smooth walls (Fig. 2), the apical portion of which was tapered (Fig. 3) or slightly curved (Fig. 4); only occasionally did it wrap around itself like a spring (Fig. 5). It is worth noting the presence of some ovoid plaque-like areas near the apex, apparently lying outside the true and proper wall (Figs 3 and 4).

In this strain, the innermost portion of the sample shows abundant microconidia and occasional macroconidia. The microconidia (or microaleuriospores) are small (2·8–3 μm long and 1·8–2 μm wide), tear- or peg-shaped; they can be borne singly along the sides of hyphae or can be grapelike bunches (Fig. 6). The macroconidia (or macroaleuriospores) are larger (11–13 μm long and 3–6 μm wide), cylindrical and pencil-shaped and often develop directly at the ends of thick hyphae (Fig. 7). Characteristically they have a smooth surface and contain several septa, which are clearly visible in the forming conidia (Fig. 8).

The 96-h-old control mycelium often presents barrel-shaped arthroconidia (Fig. 9), i.e. the parasitic conidia of these fungi. In addition, the innermost portion of these samples has amorpho-fibrillar extruded material which is, at times, abundant (Fig. 10) and which is not seen in the 24-h samples.

Under TEM the ultrastructure of *T. rubrum* can be compared to that of other species in this genus. The organelles such as the nuclei (Fig. 11), mitochondria, septa and reserve material (glycogen) all appear normal (Fig. 12). One characteristic of this fungus which has not been observed in other dermatophytes is the presence of a membrane-like structure located in the outer portion of the wall (Fig. 12). This 'membrane' appears as a discontinuous bi-stratified, at times shiny, layer running along the hyphae (Fig. 13).

After 24 h of contact with G8 the fungus showed numerous mutations: while the shape of the apex was still normal, the walls were no longer smooth but, rather, covered with a material which appeared extruded from the wall itself (Fig. 14). Micro- and macroconidia were no longer present; on the contrary, forms typical of resistance (the chlamydospores) appear; these can be recognized by their rounded shape and diameter (7–8 μm), much larger than that of the hyphae with which they are associated (2 μm) (Fig. 15); the walls of chlamydospores are depressed at several points and, as in the hyphae, covered with small granules of extruded material. There are significant alterations in the organelles, the most highly affected being the mitochondria that have a round shape with crests of the internal membrane showing no regular organization.

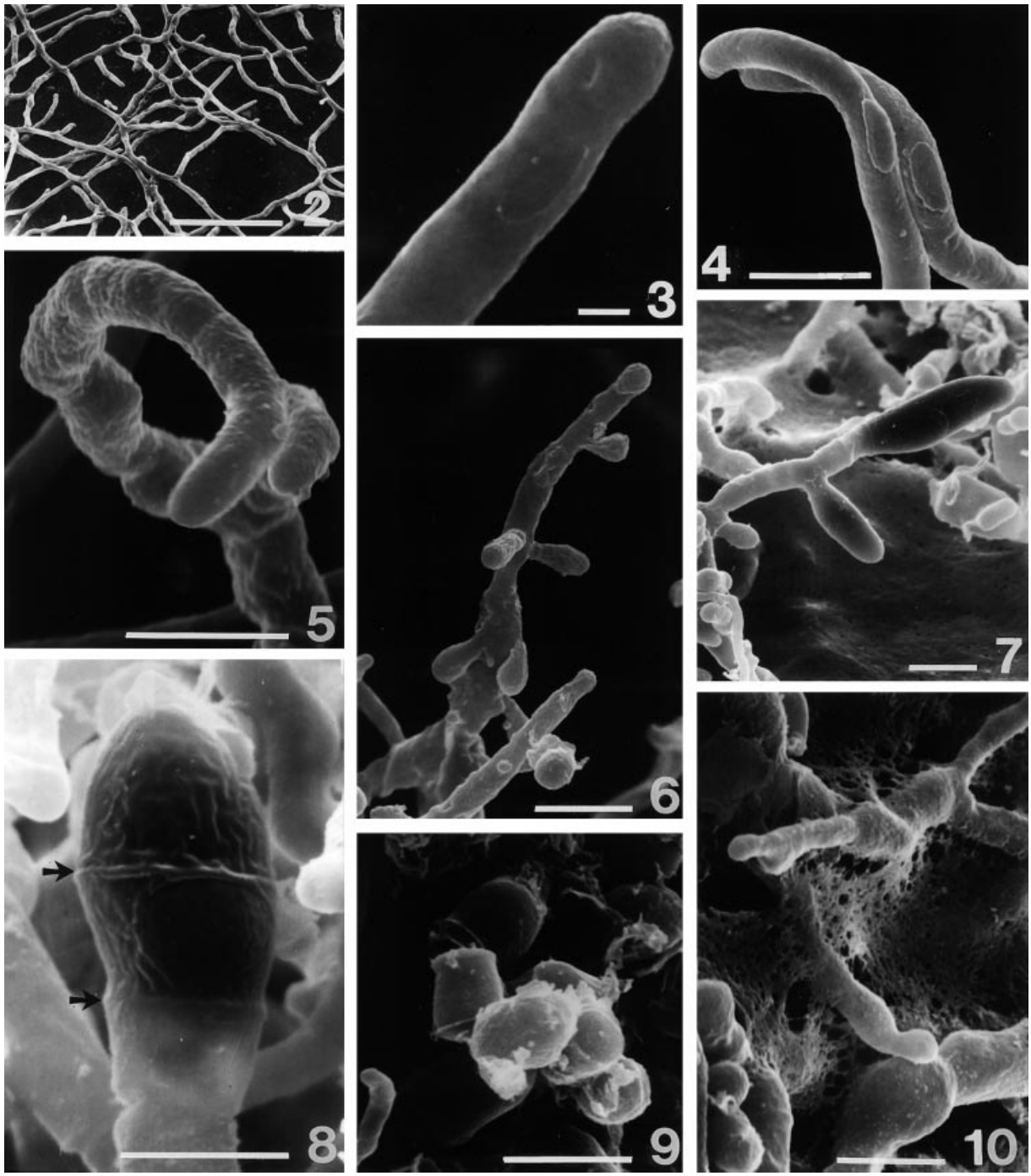



**Fig. 2** General appearance of control *Trichophyton rubrum* mycelium. SEM. Scale bar = 50 $\mu$m.
**Fig. 3** Same sample: detail of straight apex with smooth wall. SEM. Scale bar = 1 $\mu$m.
**Fig. 4** Same sample: two *Trichophyton rubrum* control apices showing a curved shape and evident supraparietal plaques. SEM. Scale bar = 1 $\mu$m.
**Fig. 5** Same sample: with apices bent over probably forming a spiral organ. Scale bar = 5 $\mu$m.
**Fig. 6** Control *Trichophyton rubrum*: conidiophore branches bearing numerous pyriform microconidia. SEM. Scale bar = 5 $\mu$m.
**Fig. 7** Control *Trichophyton rubrum*: in the innermost part of the mycelium among the microconidia there are two large macroconidia. SEM. Scale bar = 5 $\mu$m.
**Fig. 8** Control *Trichophyton rubrum* at 24 h: a macroconidia being formed can be seen with well-marked septa (arrows). SEM. Scale bar = 5 $\mu$m.
**Fig. 9** Arthroconidia of control *Trichophyton rubrum* at 96 h showing the characteristic barrel-shape. SEM. Scale bar = 5 $\mu$m.
**Fig. 10** The same sample in which the amorphous extruded material was observed between the oldest innermost hyphae. SEM. Scale bar = 5 $\mu$m.

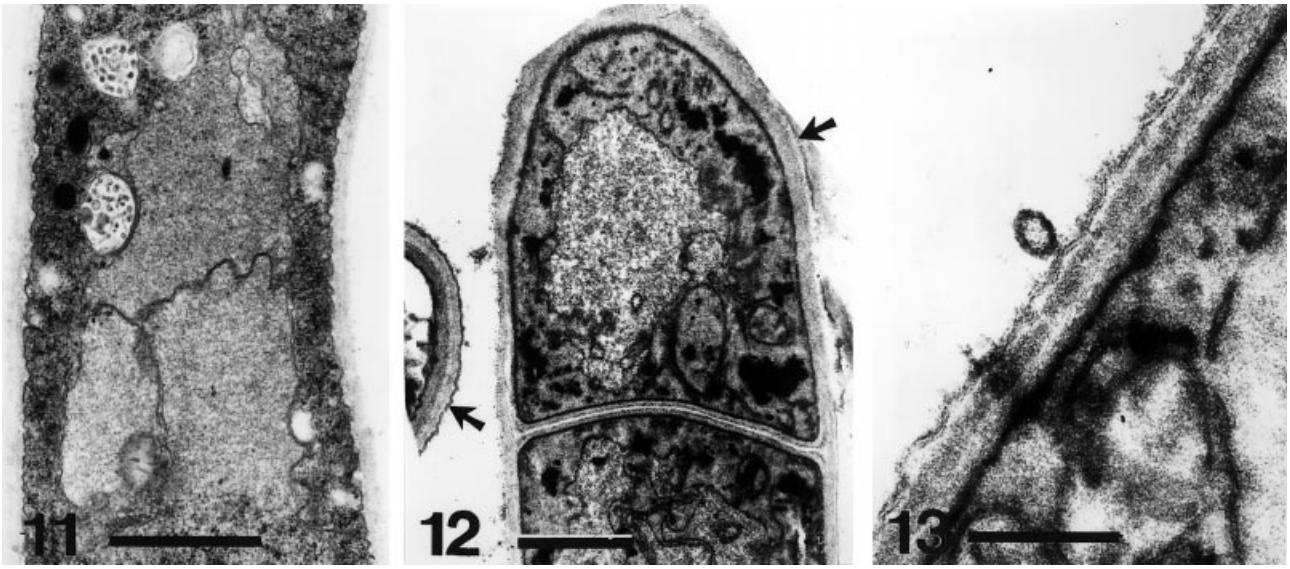



**Fig. 11** Control *Trichophyton rubrum* at 24 h: normal endomembrane system including that of the nuclear envelope. TEM GA-osmium. Scale bar = 1 $\mu$m.
**Fig. 12** Detail of hypha with normal septum, wall and organelles. Note the 'membrane' outer to the wall (arrows). TEM GA-permanganate. Scale bar = 1 $\mu$m.
**Fig. 13** Same sample where greater magnification shows the outermost layer of the wall, similar to a two-layer membrane. The internal organelles are normal. TEM GA-permanganate. Scale bar = 0·5 $\mu$m.

The vacuoles are numerous and vary greatly in size. There is an abundance of microbodies, surrounded by a single membrane and having the protein matrix of average electron density; there is a great deal of extruded material of varying consistency located along the walls (Fig. 16). The endomembrane system is particularly affected by G8 treatment. Greater magnification shows a break-up of the plasmalemma through which there is a releasing of cytoplasm; there are also evident vacuolar tonoplast ruptures at some points and décollement between the nuclear envelope membranes (Fig. 17).

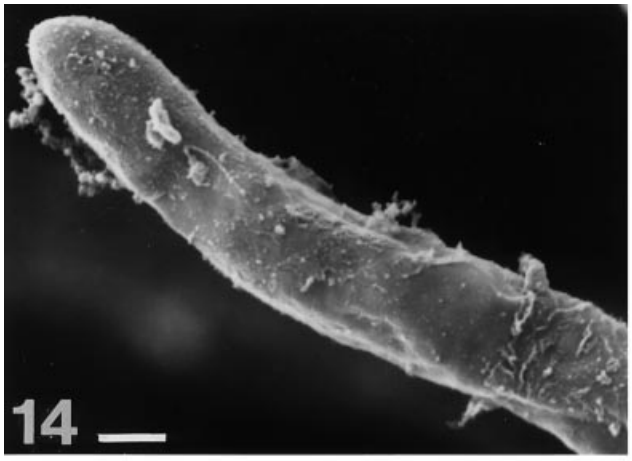
After 96 h of treatment, besides the findings encountered at 24 h, one also finds an abnormal narrowing of the hyphal tubes and an increase in the amount of extruded materials between the hyphae (Fig. 18). TEM shows an increase in the autolytic phenomena reaching total cytoplasm digestion in some hyphae (Fig. 19). The outer 'membrane' often appears to be in relief, raised up and separated from the wall by a more electron-transparent layer (Fig. 20) or it may even be stripped away, broken up and dispersed in the outside medium (Fig. 21).

## Discussion

The new compound 4-amino-3-methyl-1-phenyl-pyrazolo-(3,4-*c*)isothiazole, G8, has proved to inhibit the growth of this strain of *T. rubrum* well. Indeed, this molecule had already proved active on some dermatophytes [3]. Although it can be asserted that the minimum inhibiting concentration (MIC) for *T. rubrum* lies between 100 and 200 $\mu$g ml$^{-1}$, it was not possible achieve the minimum lethal concentration (MLD), even at extremely high doses.

Ultrastructural findings for G8-treated *T. rubrum* clearly show that the substance targets the cell membrane. After just 24 h of treatment the plasmalemma and organelle membranes were broken up or altered. This shows that G8 is similar to other azoles (i.e. oxiazole, thiazole, imidazole, pyrazole and triazole) which upset the normal synthesis of ergosterol, an essential membrane component in most fungi, thus leading to an accumulation of some methylate precursors and the subsequent break-up of the membrane itself [7].

Another interesting finding is that, following treatment, saprophytic micro- and macroconidia were suppressed: while present in the controls they could no longer be seen in the treated samples. In addition, swollen resistance forms, i.e. chlamydospores, with thickened walls were induced. These forms could be what enables the fungus to restore itself when good conditions return and may explain why MLD was not reached with this substance, even at the highest concentrations.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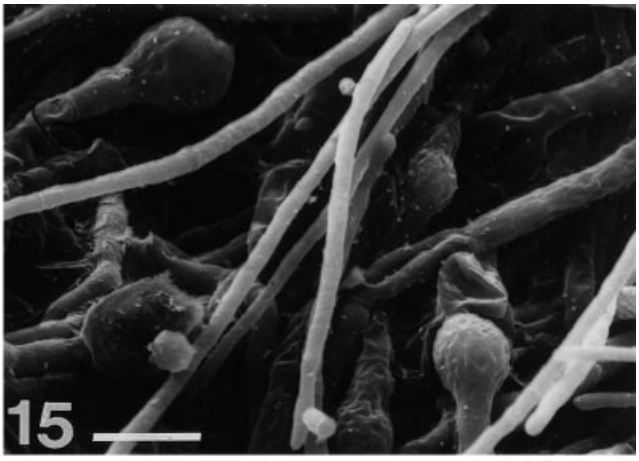
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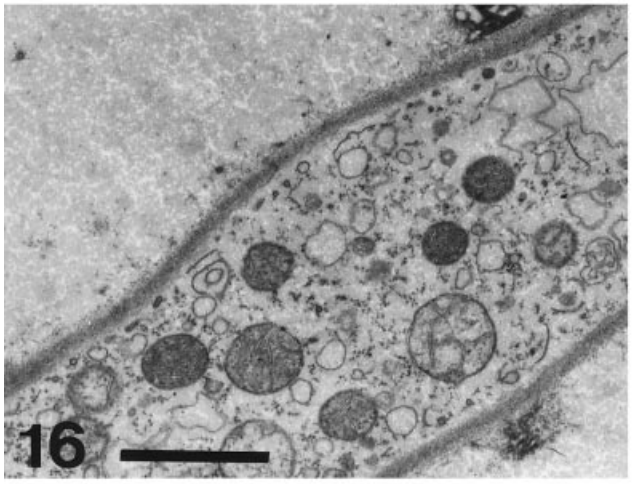
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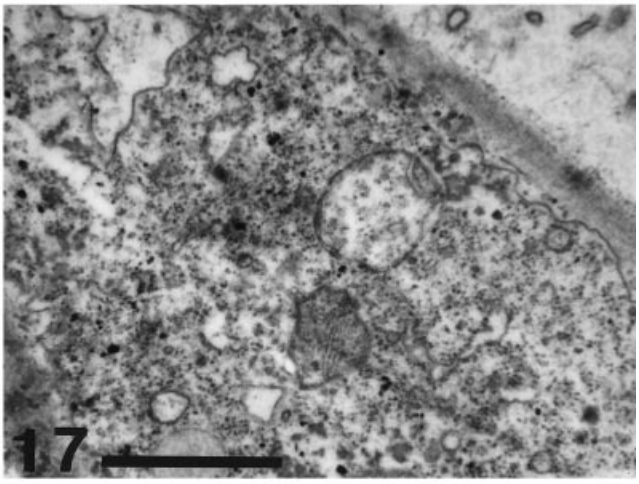
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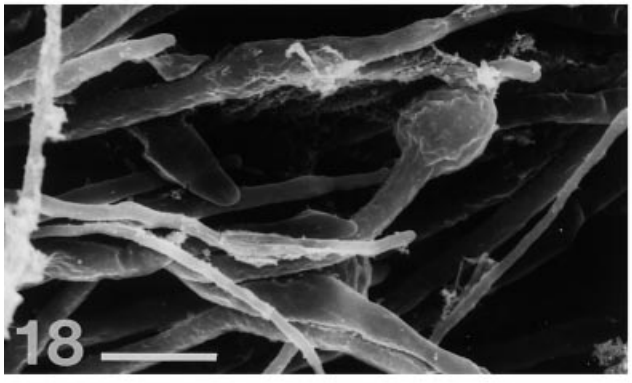
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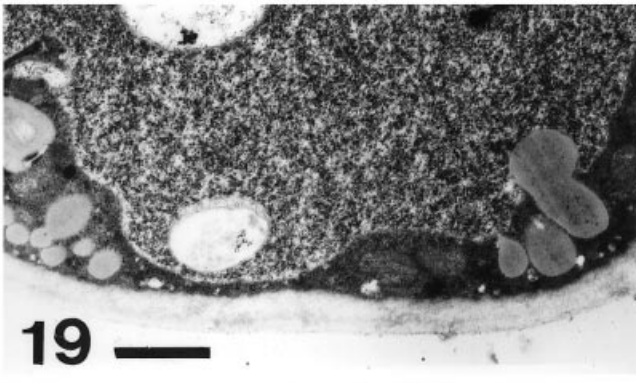
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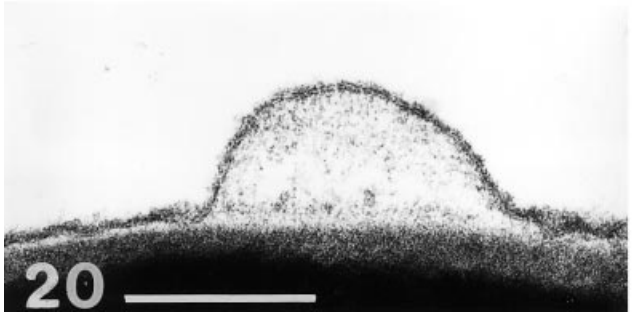
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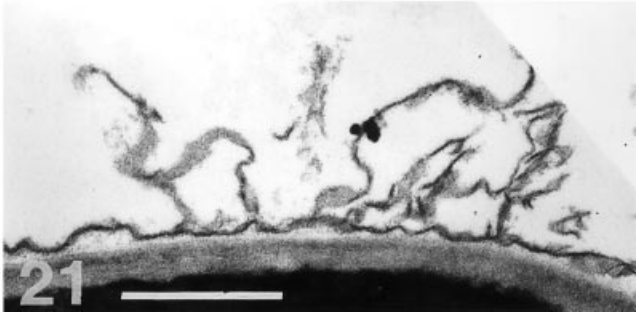
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**Fig. 14** Apex of *Trichophyton rubrum* treated for 24 h with G8: the wall is covered with varying-sized granules and/or heaps of extruded material. SEM. Scale bar = 1 $\mu$m.
**Fig. 15** Same sample: large chlamydospores are evident with corrugated walls covered with extruded granules. Scale bar = 10 $\mu$m.
**Fig. 16** *Trichophyton rubrum* treated for 24 h with G8: under TEM the endomembrane system and the organelle envelopes appear highly damaged and broken up at several points. The mitochondria, in particular, appear abnormal in shape and are all rounded. TEM GA-osmium. Scale bar = 1 $\mu$m.
**Fig. 17** Same sample under greater magnification: note that the membrane of the nuclear envelope is stripped away and that the cytoplasm is leaking out due to rupture of the plasmalemma. TEM. GA-osmium. Scale bar = 1 $\mu$m.
**Fig. 18** *Trichophyton rubrum* treated for 96 h with G8: abnormal hyphae with variable section, chlamidospores and extruded material are visible. SEM. Scale bar = 10 $\mu$m.
**Fig. 19** Same sample under TEM in which an autophage process is evident. Scale bar = 1 $\mu$m.
**Fig. 20** *Trichophyton rubrum* after 96 h of treatment. The outer 'membrane' is raised and separated from the wall by a more electron-transparent layer. TEM GA-permanganate. Scale bar = 0·5 $\mu$m.
**Fig. 21** Same sample: in some areas the outer 'membrane' is stripped away from the remainder of the wall and dispersed in the medium. TEM GA-permanganate. Scale bar = 1 $\mu$m.

Finally, but not less important, is the ultrastructural morphology of this fungus which had not been studied previously even though it is one of the most clinically important dermatophytes. In particular, the plaques on the outer wall of the hyphal apex and the coiled spiral organs observed under SEM could, in our view, be systems useful in the initial approach to parasitizing the host. The outer 'membrane' is particularly interesting; it could easily be observed under TEM with normal contrast medium and was found both in untreated samples and in those treated with the various concentrations of G8; in spite of that, no mention could be found in the bibliography. It can be assumed that this membrane constitutes the main obstacle to fixative penetration, which forced us to review some of the steps in the normal TEM preparation procedure widely applied with other dermatophytes [5,6]. Nevertheless, that this is in effect a membrane is also demonstrated by the fact that, following G8 treatment, it breaks up in the medium. Affecting the membrane as a whole, isotiazole may have acted by breaking down not only the endomembrane system but also that of the outer membrane, causing its separation and release into the medium. A logical consequence of such membrane break-up is the unusual extrusion of material seen as granules deposited on the surface of the walls and amorphous material between the hyphae.

Therefore, in view of the marked fungistatic capacity of G8 and its action mechanism – which appears similar to that of other azoles on the market – this new molecule can be considered quite useful as an efficient antidermatophyte of the future.

## Acknowledgements

This work was supported by grants from the Consiglio Nazionale delle Ricerche (CNR), Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST) of Italy.

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