Morphological changes of the dermatophyte *Trichophyton rubrum* after photodynamic treatment: a scanning electron microscopy study

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Treatment strategies for superficial mycosis caused by the dermatophyte *Trichophyton rubrum* consist of the use of topical or oral antifungal preparations. We have recently discovered that *T. rubrum* is susceptible to photodynamic treatment (PDT), with 5,10,15-tris(4-methylpyridinium)-20-phenyl-[21H,23H]porphine trichloride (Sylsens B) as a photosensitizer. The susceptibility appeared to depend on the fungal growth stage, with PDT efficacy higher with microconidia when compared to mycelia. The aim of this study was to investigate, with the use of scanning electron microscopy, the morphological changes caused by a lethal PDT dose to *T. rubrum* when grown on isolated human stratum corneum. Corresponding dark treatment and light treatment without photosensitizer were used as controls. A sub-lethal PDT dose was also included in this investigation. The morphologic changes were followed at various time points after the treatment of different fungal growth stages. Normal fungal growth was characterized by a fiber-like appearance of the surface of the hyphae and microconidia with the exception of the hyphal tips in full mycelia and the microconidia shortly after attachment to the stratum corneum. Here, densely packed globular structures were observed. The light dose (108 J/cm²) in the absence of Sylsens B, or the application of the photosensitizer in the absence of light, caused reversible fungal wall deformations and bulge formation. However, after a lethal PDT, a sequence of severe disruptions and deformations of both microconidia and the mycelium were observed leading to extrusion of cell material and emptied fungal elements. In case of a non-lethal PDT, fungal re-growth started on the remnants of the treated mycelium.

**Keywords** *Trichophyton rubrum*, antifungal, photodynamic treatment, SEM, morphology

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**Introduction**

Superficial dermatomycosis caused by the dermatophyte *Trichophyton rubrum* is one of the most common human skin infections worldwide [1–4]. The treatment often consists of the use of a topical or an oral application of an antifungal drug [5–7] or a combination of both modes of therapy [8]. The frequently used drugs can be divided into three groups, i.e., polyenes, azoles and allylamines [9,10]. Apart from these groups, griseofulvin [9] and ciclopiroxolamine [11] are occasionally employed to treat these infections. Furthermore efforts have been made to use photodynamic treatment (PDT) for dermatophyte infections [12–14]. We have recently demonstrated that *T. rubrum* is susceptible, both *in-vitro* [15] and *ex-vivo* [16], to PDT...
with a synthetic porphyrin photosensitizer, Sylsens B, the cationic porphyrin 5,10,15-tris(4-methylpyridinium)-20-phenyl-[21H,23H]-porphine trichloride (see Fig. 1). PDT refers to the use of light-activated agents, called photosensitizers, in combination with light of an appropriate wavelength and molecular oxygen [17]. This photochemical reaction results in the production of reactive oxygen species, namely singlet oxygen (1O2) and superoxide anion radical (O2−). As a consequence, the photodynamic effect can cause injury to cells of different kind of pathogens if they are in close proximity to the photosensitizer [18], which in turn contributes to an effective treatment for localized infections [19]. It is generally agreed that 1O2 is the key agent responsible for the cellular damage during PDT. However this reactive oxygen has a short life time and hence its diffusion distance is very small (<200 nm) [20]. A selective, tight binding of the photosensitizer to the target microorganism is thought to be necessary for PDT effectiveness [21–23].

Several studies concerning the action mechanisms of antifungals have been published [9,24–27]. Some of the morphological studies, including those involving T. rubrum have demonstrated degenerative changes of fungal mycelia and spores [9,28–32]. However, there have been few reports describing morphological changes caused by PDT to microbes in general [33,34] and to T. rubrum, in particular.

In the present work we used scanning electron microscopy (SEM) to examine the effect of a lethal PDT dose on T. rubrum grown on human stratum corneum (SC) using Sylsens B as a photosensitizer. Special attention was paid to the normal T. rubrum morphology under the selected conditions. Since a thorough binding of photosensitizer to the fungus is essential for successful PDT treatment, we focused on its effects in causing morphological alterations of fungal walls. We investigated not only the effect of the PDT lethal dose, but also, under similar conditions, the morphological changes caused by Sylsens B in the dark and the effect of the light dose alone on the fungal growth. In addition, we visualized the growth of this dermatophyte after sub-lethal PDT doses.

The application of the different treatments was performed on a novel ex-vivo model developed by the present authors [16]. Since our previous studies revealed that the susceptibility of T. rubrum to PDT with Sylsens B depended on the fungal growth stage [22], it was decided to visualize the morphological changes at various growth stages and at different time points after the treatment. For the different treatments the selected fungal growth stages corresponded to 8, 48 and 72 h after spore inoculation on isolated human stratum corneum (SC) present in the ex-vivo model [16]. Under the given conditions, the 8-h growth stage was characterized by germinating microconidia and the absence of fungal hyphae. At 48 h after spore inoculation, hyphal growth started, while the 72-h growth stage was represented by the full mycelium.

**Materials and methods**

**Materials**

The fungus T. rubrum was purchased from the Centraalbureau voor Schimmelcultures (CBS, strain no: 304.60), Utrecht, The Netherlands. For the preparation of a microconidia suspension, T. rubrum cultures were grown on Sabouraud dextrose agar (SDA; Sigma-Aldrich Chemie, Germany) at room temperature. Sylsens B (mol wt: 769.16 g/mol) was synthesized by Buchem Holding BV (Lieren, The Netherlands; purity was more than 99% as determined with NMR). Glutaraldehyde and OsO4 were purchased from Electron Microscopy Sciences (Hatfield, Great-Britain), the cacodylate buffer from Sigma (Sigma-Aldrich Chemie, Germany), while all other chemicals were purchased from J.T.Baker (Deventer, The Netherlands). Stock solutions of 6 mM Sylsens B were prepared and stored at 4°C for ≤1 week.

**Preparation of the microconidia suspensions**

The protocol for obtaining a suspension of microconidia produced by T. rubrum grown on SDA was

Fig. 1 Chemical structure of the porphyrin photosensitizer 5,10,15-tris(4-methylpyridinium)-20-phenyl-[21H,23H]-porphine trichloride (Sylsens B).
described previously [16,35]. The microconidia suspensions (10–40 × 10⁶ colony forming units (CFU)/mL) were stored in liquid nitrogen for no longer than 6 months. Counting colonies on malt extract agar (MEA) plates was used as a viability check.

Preparation of the human SC

Abdominal or breast skin collected following cosmetic surgery was obtained from a local hospital. After removal of the fat tissue, the skin was cleaned with distilled water and dermatomed to a thickness of approximately 250 µm using a Padgett Electro Dermatome Model B (Kansas City, USA). The dermatomed skin was incubated on the dermal side with a 0.1% trypsin solution in phosphate buffered saline of pH 7.4 (4°C) overnight. After 1 h at 37°C, the SC was removed manually, dried in the air for 24 h and then kept under nitrogen over silica gel for no longer than 3 months.

Light source

Illuminations were performed with a lamp from ‘MAS-SIVE’ (no.74900/21), 1 x max.500W-230 V-R7s, IP 44. To avoid heating of the samples during illumination, a 5 cm water filter was used to absorb infrared radiation. Light intensity was measured with an IL1400A photometer equipped with a SEL033/F/U detector (International Light, Newburyport, MA, USA). A red cut-off filter at 600 nm was used to obtain the red portion of the spectrum produced by the lamp. The light emitted by the lamp had a wavelength range of 580–870 nm. The irradiance at the sample of the infected human SC was 30 mW/cm².

The ex-vivo model and photodynamic treatment

For the photodynamic, dark and light treatment, 15 µl of a T. rubrum microconidia suspension containing 45 to 450 CFU was inoculated onto human SC in the ex-vivo model as described previously [16]. Before irradiation, the pieces of SC and microconidia were kept in an incubator at 28°C. The different treatments were performed in a water incubation medium of pH 5.2 supplemented with Sylsens B (with the exception of the light treatments). As described in the ex-vivo model, the membrane filters containing the SC and the treated fungal elements were transferred after every treatment to a malt extract agar (MEA) plate and incubated at 28°C [16].

The fungus was subjected to the treatments at 8, 48 and 72 h after inoculation with microconidia. Twenty µM Sylsens B was used in the case of PDT or dark treatment carried out 8 h after inoculation. Two hundred µM Sylsens B was used for PDT or dark controls performed at 48 and 72 h after inoculation. Previous studies reported that PDT applied with 200 µM Sylsens B 72 h after inoculation resulted in 80–90% of the cases in complete fungal inactivation. In the remaining cases we observed one or two fungal colonies appearing 5 days after the treatment [22]. The described situation was defined as a sub-lethal PDT dose. A light dose of 108 J/cm² was applied during all photodynamic and light treatments.

Scanning electron microscopy

In order to investigate structural alterations of T. rubrum following different treatments, scanning electron microscopy was applied to visualize the (treated) fungus located on the sheets of human SC. Inoculum from untreated T. rubrum on human SC, in different growth stages, were used as control. The samples were examined at 1, 20 and 40 h after the treatment or at 8 days after inoculation. A JEOL JSM-6700F field emission SEM (Tokyo, Japan) was used at an acceleration voltage of 5 kV.

The SC sheets were fixed overnight at 4°C in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) and post-fixed on ice for 1 h in 1% OsO4 (in 0.1 M sodium cacodylate buffer of pH 7.2). Subsequently, the samples were rinsed (3 times) with PBS and dehydrated in increasing concentrations of ethanol (50–100%). The samples were then critical-point dried with CO2 using a Baltec CPD 030 (Balzers, Lichtenstein) and coated with gold-palladium using an Emitech K650X sputter coater (Ashford, England). Every experiment at a specific growth stage and exposure was repeated at least 4 times (4 duplicates within every experiment) and representative images were selected (from approximately 150 to 200 for every condition and corresponding growth stage). Results were summarized and scored by three individuals (one of whom was blinded to the experimental procedures).

Results

The results of the PDT treated samples, dark and light controls, at different time points after spore inoculation, are presented and compared to normal fungal growth. The score results of the structural fungal wall alterations after the different treatment modalities are summarized in Table 1.

Eight hours after microconidia inoculation

SEM images of the T. rubrum growth stage corresponding to 8 h after spore inoculation are shown in Figs. 2...
Table 1  Structural fungal wall alterations\(^1\) observed as a result of PDT, light only treatment (108 J/cm\(^2\)) and dark treatment with the photosensitizer Sylsens B alone of *Trichophyton rubrum* applied at 8 (A), 48 (B) and 72 (C) hours after spore inoculation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Minor wall deformations</th>
<th>Bulge formation</th>
<th>Smooth wall appearance (loss of fiber-like structure)</th>
<th>Extrusion of cell material</th>
<th>Flattening</th>
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<tr>
<td></td>
<td>I(^2)</td>
<td>II(^3)</td>
<td>I</td>
<td>II</td>
<td>I</td>
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<td>(A)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>100</td>
<td>45</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
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<td>18</td>
<td>Sporadically</td>
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<tr>
<td>Light control</td>
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<td></td>
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<td>0</td>
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<tr>
<td>None</td>
<td>0</td>
<td>6</td>
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<td>(B)</td>
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<td>30</td>
<td>28</td>
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<tr>
<td>Dark control</td>
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<tr>
<td>Light control</td>
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\(^1\)The number of observed alterations is expressed as the percentage of the number of fungal elements scored in total. In total 60 to 80 fungal elements from different preparations were scored.

\(^2\)Eight hours after treatment.

\(^3\)Eight days after spore inoculation.
Fig. 2  SEM images showing *Trichophyton rubrum* microconidia 8 h after spore inoculation on human SC. In (A), a number of microconidia, just germinated, are depicted. Clearly shown are the adherences of the microconidium and germ tube to the SC can (white arrows). In (B), the regular globular structure of the microconidia wall is noticed, while the wall structure in (C) (after microconidium germination) has a more fiber-like appearance. The white arrow points to the germ tube.

Fig. 3  SEM observations of *Trichophyton rubrum* microconidia after PDT (20 μM Sylsens B, 108 J/cm² of red light, series A), dark treatment (20 μM Sylsens B, series B) and light treatment alone (108 J/cm² of red light, series C), applied at 8 h after spore inoculation on SC. (A1) 1 h after PDT. Notice the disintegration of the microconidium head and the extrusion of internal material (black arrow). (A2) 20 h after the PDT. Notice (white arrows) the proceeding process of disruption of the outer wall, including the loss of the fiber-like appearance. (A3) Eight days after spore inoculation. The remains of a *T. rubrum* microconidium, following PDT shortly after the germ tube development, completely emptied. (B) 1 h after the dark treatment. Notice the small bulge formation (black arrow). (C) 1 h after the light treatment. Here minor wall deformations can be noticed, such as indented wall (black arrows).
and 3. At this time point, the microconidia were attached to the SC and the germination process had started (Fig. 2).

Application of PDT with 20 μM Sylsens B (known to cause complete fungal inactivation [22] resulted in wall deformations and leakage of internal cell material shortly after the treatment (Fig. 3, A1 and A2). Seven days later, only the remains of the successfully treated germlings could be observed (Fig. 3, A3). Control dark treatment with the same Sylsens B concentration resulted in minor wall deformations such as bulge formation (Fig. 3B). Treatment with light alone (108 J/cm²) at 8 h after spore inoculation resulted in minor wall deformations, such as indented fungal walls (Fig. 3C). Later fixation times showed fungal recovery in the case of dark or light treatment controls. This indicates that the observed changes in the fungal wall are reversible.

Figure 4 provides a typical example of fungal growth at eight days after spore inoculation under ex-vivo conditions in the absence of PDT. There was full and intense mycelium growth and similar development was noted after a dark or light treatment only, indicating again that the morphological alterations due to dark or light treatment (observed shortly after the treatment) are not permanent, with normal fungal growth was observed at eight days after spore inoculation.

Forty-eight hours after microconidia inoculation

Figures 5 and 6 present the appearance of the fungus at this time and as can be noted, there is full hyphal proliferation. In Fig. 5 we focused attention on the appearance of the outer hyphal walls at this proliferation stage. The walls resembled that of a fiber mesh, resulting in a rough appearance. At 1 and 20 h after the lethal PDT dose, bulge formation and a ruptured wall were observed (Fig. 6, A1 and A2) as well as small smooth appearing hyphal wall areas (inlay in Fig. 6, A2). Here, the loss of the wall fibers had started and at a later time point (40 h after the treatment) and in extreme cases the fiber-rich structure disappeared, rendering a completely smooth appearance to the outer wall (Fig. 6, A3). Eventually, after the successful PDT treatment (8 days after the original spore inoculation), flattened and dented hyphae were observed (Fig. 6, A4a and A4b).

Occasionally, we observed severely damaged fungal elements after treatment with 200 μM Sylsens B in the dark (Fig. 6-B). However, in most cases, dark treatment resulted in similar (but less severe) deformations, while T. rubrum hyphae treated with 108 J/cm² of red light alone had an almost normal appearance (Fig. 6C).

Seventy-two hours after microconidia inoculation

The growth of T. rubrum on SC in the ex-vivo model at 72 h after spore inoculation can be seen clearly in Fig. 7. In Fig. 7A the full mycelium is clearly shown and sporulation of the dermatophyte had occurred (see Fig. 7B and C). Of particular interest is the difference in fungal wall structure in the middle of the hyphae compared to the hyphal tips (Fig. 7D and E). The tips were more densely packed with...
globular appearing material a difference that could not be observed in the other growth stages. The morphology of the hyphal wall in the branching areas is shown in Fig. 7F. Close inspection of the branching point showed a densely packed, globular structure.

The application of a lethal PDT dose at 72 h after spore inoculation resulted not only in fiberless fungal hyphae (1 h after the treatment, Fig. 8, A1a and A1b), flattened and emptied mycelium structures (Fig. 8, A2) 20 h after the treatment, but also in other time related morphological changes. Worth mentioning is the abundant bulge formation, observed 8 days after the original spore inoculation (5 days after PDT), on the already smooth appearing hyphae (Fig. 8, A3). As a result of the PDT treatment, the hyphae were deprived of their fiber-like, grainy wall material giving them a smooth appearance.

The dark treatment (Fig. 8, B) caused some wall deformations, while after light treatment hardly any changes could be observed (Fig. 8, C). From both images in Fig. 9, it is clear that in the case of an ineffective PDT dose, fungal re-growth started on the remains of the fungal mycelium. As can be observed during a close inspection, re-growth appeared to start not only on the growing tips but also in other areas of the ‘old’ fungal hyphae.

Discussion

Dermatophytes exhibit various morphologies depending on their growth stage and environmental conditions [36]. According to Vasquez et al., the surface of micro- and macroconidia of members of the genus Trichophyton, when cultivated under in-vitro conditions, appears to be smooth. Bibel et al. described the ruffled surface of Trichophyton mentagrophytes when cultivated in-vitro on Sabouraud dextrose agar [37], while Osumi described a granular surface to the cell wall of T. rubrum [38]. In our studies, the surface
of both the germinated microconidia and the hyphae had a rough, granular and fiber-mesh-like appearance. Shortly after the microconidia adherence to the SC (but before germ tube development) the spore wall appeared to be more globular with a lack of fiber-like material and coated with a clear membrane (see Fig. 10 to illustrate this difference). This different structure might be required to enable attachment to the SC [39]. The fungal wall structure on hyphal tips also differed from the rest of the hyphae, i.e., a densely packed globular structure. We noticed this typical structure of the tips only at 72 h after spore inoculation. This might indicate an increased metabolic activity at the tip of the hyphae.

In this study, we showed that morphological damages occurred not only after PDT treatment but also after exposure to Sylsens B in the dark and to a lesser degree after light treatment without the photosensitizer. The similarities for the different growth stages are discussed below.

In general, in all fungal growth stages, the observed damage due to the different treatments occurred shortly after the treatment. This is, in the case of PDT, to be expected because of the short lifetime of the reactive $^1O_2$. Although similar damage was observed after all treatments, only after PDT was the extent and severity of the damage such that it resulted in fungal death. The main consequences of an effective PDT dose were indented and deformed fungal elements, bulge formation, rupture of the fungal hyphae and, eventually, the development of areas displaying leakage of internal cell material. This sequence of events must have been caused at first by both the binding of the cationic porphyrin Sylsens B in the dark (during the incubation) to the fungal wall, followed by the production of $^1O_2$ after light application. Indeed, from the results of the dark treatments, minor bulges and leakage formation was observed, giving rise to some decrease in wall stability. Binding of Sylsens B in the dark to the fungal wall could decrease the binding of other cationic molecules involved in wall stability and transport across the cell. This could also explain the growth delay after dark treatment with Sylsens B and the dark toxicity sporadically observed in earlier studies when Sylsens B was used at 200 µM concentrations [16]. Treatment with 108 J/cm² of red light alone (i.e., no photosensitizer) however, did not cause a growth delay in our previous experiments [16]. This
indicates that the observed injury induced by the red light had only a minor influence on fungal life.

As regards the bulge formation on the fungal hyphae after a dark treatment (and occasionally after a light treatment), it must be mentioned that similar structure alterations we also noticed (sporadically) on untreated fungal hyphae growing on human SC in the ex-vivo model.

We also looked more closely at the effect of only partly effective PDT on fungal structure (Fig. 9). The SEM study confirmed our previous observation that the unsuccessful PDT (80–90% fungal kill) caused an arrest of the fungal growth [22]. However, the fungal body was able to recover at certain surviving regions of the hyphae. As illustrated in Fig. 9 re-growth was observed at hyphal tips and in other hyphal areas. These observations can be of practical importance to PDT treatment of T. rubrum applied at the time of full mycelium growth. The metabolically active fragments of hyphae are probably more resistant to PDT, either because of their better anti-oxidative defense due to the altered binding capacity for Sylsens B, or because of an increased keratinase enzyme production during this growth stage. Recently it was shown that the inhibition of keratinase activity increased PDT efficacy when applied on T. rubrum mycelium at 72 h after spore inoculation [22]. In the present study, we also demonstrated a difference in wall structure of the hyphal tips of the full mycelia at 72 h after spore inoculation when compared to the rest of the fungal hyphae and the tips during earlier stages. A consequence of these structural differences may be a decreased binding capacity of Sylsens B to these hyphal tips. This may result in lower local PDT effect and, consequently, the observed fungal re-growth. A similar low binding capacity for Sylsens B might be present in the areas of hyphal branching. Our SEM study indicated a difference in wall structure in branching areas (more globular and dense) compared to other hyphal regions. Similar morphologic differences have been noted in other studies [40,41].

Fig. 8 Observation of Trichophyton rubrum by means of SEM after PDT (200 μM Sylsens B, series A), dark treatment (200 μM Sylsens B, series B) and a light only treatment (108 J/cm² of red light, series C), applied at 72 h after spore inoculation on SC. (A1) 1 h after the PDT. Notice in both hyphae close-ups (A1a and A1b) the alterations in the fungal wall appearance due to the PDT. (A2) 20 h after the PDT. Notice at this time the appearance of flattened and emptied hyphae. (A3) Eight days after spore inoculation. Notice the almost smooth appearance of the fungal hyphae due to dissolution of the membrane particles, the bulges and the flattened and fiberless hyphae. (B) 20 h after the dark treatment. Notice both normal and damaged fungal hyphae. (C) 20 h after the light treatment. Notice the normal mycelium appearance.

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observations may be of importance for the treatment strategy. We hypothesize that better fungicidal effect could be achieved by application of a second treatment within 24 to 48 h, a time interval in which hyphal tip morphology could not change into more resistant globular structures. However, further study is warranted to confirm this hypothesis.

Finally, it must be mentioned that although the photosensitizer concentrations used were known (from previous work) to be lethal and this was checked again in the present study at 8 days after spore inoculation, the present research has no correlation to clinical data.

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