

## A novel *ex vivo* skin model to study the susceptibility of the dermatophyte *Trichophyton rubrum* to photodynamic treatment in different growth phases

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**Background:** Dermatophytes are fungi that can cause infections of skin, hair and nails because of their ability to feed on keratin. Superficial mycoses are among the most prevalent infectious diseases worldwide. Two important restrictions of current therapeutic options are the recurrence of the infection and prolonged treatment. This is especially true for infections caused by *Trichophyton rubrum*, a widely distributed dermatophyte. The application of photosensitizers for treatment of fungal infections is, within the field of photodynamic treatment (PDT), relatively new. Recently, we demonstrated that the porphyrins 5,10,15-tris (4-methylpyridinium)-20-phenyl-[21H,23H]-porphine trichloride (Sylsens B) and deuteroporphyrin monomethylester (DP mme) were excellent photosensitizers towards *T. rubrum* when using red light.

**Objectives and methods:** To evaluate the photodynamic effectiveness of the porphyrins in a situation that mimics the clinical situation, we developed an *ex vivo* model using human stratum corneum. This model offers the possibility of applying PDT at different time points during the germination and subsequent development of *T. rubrum* microconidia. The model was used for two different incubation media, Dulbecco's modified Eagle medium (DMEM) and distilled water.

**Results and conclusions:** We demonstrated that the PDT susceptibility of *T. rubrum* depended on the time of PDT application after spore inoculation. A decrease in susceptibility was observed with increasing time of PDT application for both photosensitizers in DMEM. Changing the incubation medium to distilled water resulted in an increased fungicidal effect for Sylsens B and in a decreased effect for DP mme. We conclude that *T. rubrum* is susceptible to PDT in a situation that mimics the clinical situation. The fungicidal effect of PDT on fungal spores is of particular importance.

Keywords: *ex vivo* model, stratum corneum, porphyrins, tinea, microconidia

### Introduction

Dermatophytes are fungi that can cause infections of the skin, hair and nails, in part because of their ability to utilize keratin. The cutaneous infections they cause (also called tinea) are among the most common infections in humans worldwide.<sup>1</sup> In the USA, 10% of the population has cutaneous fungal infections at any given time, and at least 40% will acquire this skin condition at some time in their life.<sup>2</sup> The dermatophyte *Trichophyton rubrum* causes the most common cutaneous infection in humans,<sup>3,4</sup> which can be very persistent.<sup>5</sup>

Two of the most important restrictions limiting the usefulness of common therapeutic options are the relatively high likelihood of recurrence of the infection and the need for prolonged

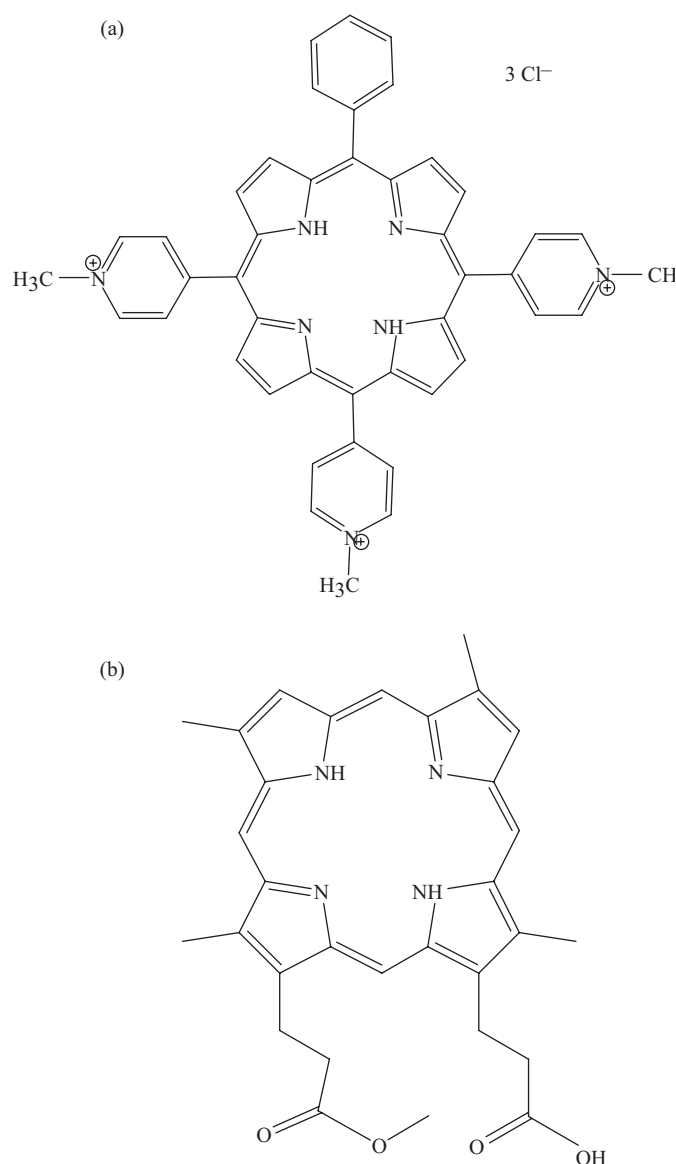
treatment.<sup>6</sup> Many current antifungal agents, such as azoles, have a fungistatic effect (a delay in growth) rather than a fungicidal effect (a complete inactivation of both fungal conidia and hyphae). Fungal conidia appear to be less susceptible to the antifungal agents than the hyphae.<sup>7,8</sup> In case of widespread, more inflammatory or persistent dermatophytoses, systemic treatment is necessary. However, when using systemic therapy one should be aware of the potential risks of drug interactions and adverse effects, such as hepatotoxicity or general drug reactions. In addition, azole resistance appears to be emerging as a serious problem in patients treated for yeast infections.<sup>9</sup>

Photodynamic treatment (PDT) refers to the use of light-activated agents called photosensitizers.<sup>10</sup> Upon irradiation with light of an appropriate wavelength, photosensitizers can initiate

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a photochemical reaction resulting in the production of reactive oxygen species, namely singlet oxygen ( $^1\text{O}_2$ ) and superoxide anion radical ( $\text{O}_2^-$ ), which can react with various cellular components. The sequence of these events is known as the 'photodynamic effect' and can result not only in a selective tissue injury, but also in the elimination of different kinds of pathogens.<sup>11</sup> The use of PDT for fungal infections is a new and promising approach.<sup>12</sup>

Recently, we have demonstrated that the porphyrins 5,10,15-tris(4-methylpyridinium)-20-phenyl-[21*H*,23*H*]-porphine trichloride (Sylsens B; Figure 1a) and deuteroporphyrin monomethylester (DP mme; Figure 1b) are excellent photosensitizers to treat *T. rubrum* in suspension when red light is used to activate them.<sup>13</sup> A single PDT *in vitro* was sufficient to achieve a 100% fungicidal effect, using either Sylsens B or DP mme.



**Figure 1.** Chemical structure of the porphyrin photosensitizers (a) 5,10,15-tris(4-methylpyridinium)-20-phenyl-[21*H*,23*H*]-porphine trichloride (Sylsens B) and (b) deuteroporphyrin monomethylester (DP mme).

To evaluate the photodynamic activity of these two porphyrin photosensitizers under conditions that mimic the clinical situation of tinea infections, we developed an *ex vivo* model using human stratum corneum (SC). This *ex vivo* model offers the possibility of applying PDT at different time points during the germination process of *T. rubrum* microconidia on human SC and also during the subsequent development of germ tubes and fungal hyphae. We have used the model to investigate the efficacy of the porphyrin photosensitizers Sylsens B and DP mme towards *T. rubrum*. The susceptibility of *T. rubrum* to PDT was investigated in different growth phases of the fungus grown under two different conditions. Because of the importance of a single treatment for tinea infections, special attention has been paid to the fungicidal effect of PDT. Information on the susceptibility of different growth phases to PDT can be of great importance for the development of a treatment strategy that would lead to inactivation of both fungal spores and hyphae. Such a treatment would prevent the recurrence of the infection and reduce the number of treatments. The growth of *T. rubrum* on human SC in the *ex vivo* model was visually investigated before and after PDT using different microscopic techniques.

## Materials and methods

### Materials

*Trichophyton 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 (Sigma-Aldrich Chemie, Germany) at room temperature. The photosensitizers Sylsens B (mol. wt: 769.16 g/mol) and DP mme (mol. wt: 524.61 g/mol) were synthesized by the Department of Bio-Organic Photochemistry, Leiden University, The Netherlands (purity determined by NMR was more than 99.5%) and kindly provided to us. Polyethyleneglycol was obtained from Genfarma B.V. (Maarssen, The Netherlands) and trypsin was obtained from Sigma (Zwijndrecht, The Netherlands), while all other chemicals were purchased from J. T. Baker (Deventer, The Netherlands). The following solvents were used: 50 mM sodium phosphate buffer pH 7.4 for Sylsens B and polyethyleneglycol/ethanol/water (3:2:5) for DP mme. Stock solutions of the photosensitizers (4.8 mM) were freshly made for each new experiment.

### Preparation of microconidia suspensions

The protocol to obtain a suspension of microconidia produced by *T. rubrum* grown on Sabouraud dextrose agar was based on the method described by Zurita and Hay<sup>14</sup> with two modifications. A 0.01% Tween 80 solution in sterile water was used instead of phosphate-buffered saline and a 7  $\mu\text{m}$  Millipore filter was used instead of a 8  $\mu\text{m}$  Nucleopore filter. The protocol was as follows: To a 14-day-old culture, 8–10 mL of a 0.01% Tween 80 solution was added. The surface was brushed with a glass rod and the resulting suspension filtered over a 7  $\mu\text{m}$  diameter filter (Millipore). The filtrate was centrifuged at 3400 g (10°C) and the resulting pellet was washed with sterile water and suspended again in sterile water in a total volume of 2–4 mL ( $10\text{--}40 \times 10^6$  cfu/mL). The obtained microconidia suspensions were

stored in liquid nitrogen for no longer than 6 months. Counting the number of colony-forming units on malt extract agar (MEA) dishes was used as a viability check. Identification of the isolated spores as microconidia was performed by the CBS.

### Preparation of the human stratum corneum (SC)

Abdomen or mammae skin was obtained from a local hospital after cosmetic surgery. After removal of the fat tissue, the skin was cleaned with distilled water and dermatomed to a thickness of approximately 250  $\mu\text{m}$  using a Padgett Electro Dermatome Model B (Kansas City, USA). The dermatomed skin was incubated at 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. The obtained SC was air-dried for 24 h and kept under nitrogen over silica gel for no longer than 3 months.

### The ex vivo model

A polycarbonate membrane filter, 25 mm in diameter and with a pore size of 2  $\mu\text{m}$  (Omnilabo, Breda, The Netherlands), was placed in the central part of a 3 cm culture dish (Greiner, Alphen aan den Rijn, The Netherlands) filled with 5 mL of MEA. A circular piece of human SC with a diameter of 1 cm was subsequently placed on the membrane filter and both were gently brushed with a paintbrush dipped in 70% ethanol. A microconidia suspension was diluted to 1000 cfu/mL and 15  $\mu\text{L}$  inoculated on the circular piece of human SC in a dish which was then placed in an incubator at 28°C. At 8, 24, 48 and 72 h after spore inoculation, PDT was applied using either Sylsens B or DP mme. The conditions of the *ex vivo* model are such that germination of the microconidia can be detected microscopically (Zeiss Axiovert 25) at 72 h after their inoculation. This implies that when PDT is applied at 72 h after spore inoculation fungal hyphae are treated. The test conditions were chosen to resemble those used in the *in vitro* experiments,<sup>13</sup> except for the duration of the incubation and the concentration of the porphyrins.

A schematic outline of the *ex vivo* model, including the PDT stadium, is provided in Figure 2.

### Light source

Illuminations were performed with a lamp from 'MASSIVE' (no. 74900/21), 1 $\times$  max 500 W-230 V-R7s, IP 44. To avoid heating of the samples during illumination, a 5 cm water filter absorbing infrared light was used. Light intensity was measured with an IL1400A photometer equipped with a SEL033/F/U detector (International Light, Newburyport, MA, USA). A red cutoff filter at 600 nm was used to obtain the red part of the spectrum of the light produced by the lamp. The light emitted by the lamp had a wavelength range of 580–870 nm. The light intensity at the level of the infected human SC was 30 mW/cm<sup>2</sup>.

### Photodynamic treatment (PDT)

At 8, 24, 48 or 72 h after spore inoculation, the membrane filter with SC containing spore inoculates was transferred from the MEA dish to a 3 cm culture dish filled with 1035  $\mu\text{L}$  of incubation medium. The incubation medium contained either 1 mL of distilled water (pH 5.2) or 1 mL of DMEM (GibcoBRL, UK)

at pH 7.4 supplemented with fetal calf serum (FCS; GibcoBRL, UK) and 35  $\mu\text{L}$  of a photosensitizer solution. Final photosensitizer concentrations were 80 and 160  $\mu\text{M}$ . To optimize the contact of the inoculated fungus with the incubation medium, the membrane filter with the SC was turned upside down during the incubation period of 2 h (see Figure 2c). The incubation was performed under continuous shaking conditions (Heidolph Shaker, unimax 2010). Shortly before the illumination period, the membrane filter containing the SC was turned back (Figure 2d). In all cases, the illumination time was 1 h using a light flux rate of 30 mW/cm<sup>2</sup> (Figure 2e). This corresponds to a light dose of 108 J/cm<sup>2</sup>. After the illumination the membrane filter with the SC was transferred to a fresh MEA dish (Figure 2f), placed in an incubator at 28°C, and fungal growth was followed for several weeks. 'Dark controls' were included, i.e. the same procedure was carried out except that the inoculated microconidia on human skin were treated with solvent or photosensitizer in the dark. In the 'light controls', the microconidia were treated with solvent in the presence of light. The efficacy of the treatment was expressed as a relative frequency of complete inactivation of fungal growth detected at day 8 after spore inoculation. To assess this a Zeiss Axiovert 25 microscope was used. If at day 8 by visual inspection no re-growth could be observed, a complete inactivation of spores and hyphae as a result of PDT was established. The treatment effect was followed for a period of several months. Every experiment contained 4 to 6 duplicates of all conditions and the experiments were repeated at least three times.

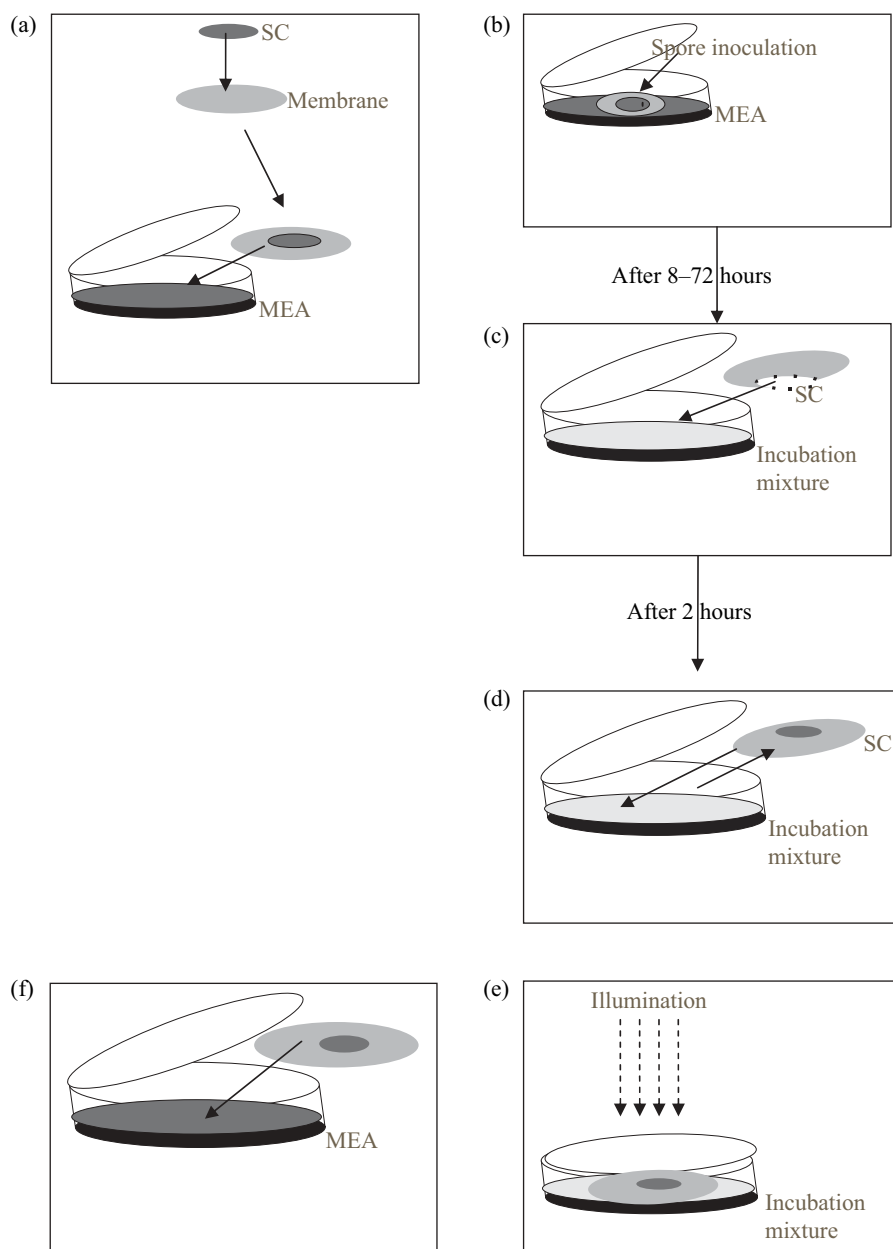
### Fluorescence microscopy

After the PDT (applied at 72 h after spore inoculation or dark treatment) the SC was placed on an object glass, washed with water, covered with a glass coverslip and examined with a Leica DM 5000 microscope equipped with differential interference contrast (DIC) optics and a digital camera (Leica DFC300FX R2 digital colour cam set). A HCX PL FLUOTAR 40 $\times$ /0.75 Ph2.objective and a HCX PL FLUOTAR 63 $\times$ /1.25 oil immersion objective were used and fluorescence images were taken using a filter cube violet + blue (H3, excitation filter BP 420–490). The pictures were taken directly after treatment with 160  $\mu\text{M}$  Sylsens B in the dark, after an unsuccessful PDT with 80  $\mu\text{M}$  Sylsens B and after a successful PDT with 160  $\mu\text{M}$  Sylsens B, when a fungicidal effect was detected. Pictures of untreated fungus, at 72 h after spore inoculation, were taken as well.

## Results

### Changing the incubation medium from DMEM to distilled water resulted in an increased PDT efficacy for Sylsens B

The results obtained with Sylsens B are provided in Table 1. When incubating in DMEM, we found that 8 h after spore inoculation, application of PDT with either 80 or 160  $\mu\text{M}$  Sylsens B resulted in a complete inactivation of the spores. At 24 h after spore inoculation under the same conditions, a fungicidal effect was obtained in 50% of the cases, while in the remaining cases only a delay in growth was observed. However, the application of PDT more than 24 h after the spore inoculation did not result in a fungicidal effect. In those cases, a growth delay of 1–2 days was



**Figure 2.** Schematic outline of the *ex vivo* model. A polycarbonate membrane filter is placed on a MEA dish and a circular piece of human stratum corneum (SC) on the membrane (a). The SC is inoculated with a microconidia suspension (b). The membrane is turned upside down and transported with the SC to the incubation medium (c). After 2 h of incubation, the membrane is turned again to allow the surface of the SC to face the illumination source (d). The illumination takes place (e). After illumination the treated SC is transferred on its membrane to a new MEA dish (f) and kept in an incubator at 28°C.

obtained, depending on the Sylsens B concentration used. Incubation in distilled water at pH 5.2 resulted in a considerably higher number of fungicidal effects compared with incubation in DMEM. When 160  $\mu$ M Sylsens B was used we obtained a fungicidal effect not only after 8 h, but also after 24 and 48 h. At these time points the fungicidal effect was almost 100%. In addition, even at 72 h after spore inoculation, when fungal hyphae were treated, we found a high percentage (65%) with complete fungus inactivation. In the experiments with DMEM the light controls resulted in 10–15 cfu for every time point at 8 days after spore inoculation. Very similar results were obtained in the dark controls

with both Sylsens B concentrations. In the case of distilled water, the light controls developed 10–15 cfu for every time point at 8 days after spore inoculation and the dark controls 5–12 cfu.

#### *Changing the incubation medium from DMEM to distilled water caused a decreased PDT efficacy for DP mme*

As far as DP mme is concerned (Table 2), the results resembled those obtained with Sylsens B in DMEM (Table 1): a complete inactivation of the spores 8 h after spore inoculation and a fungicidal effect of 50–85% when PDT was applied at 24 h after spore



**Table 1.** Occurrence of a fungicidal effect after PDT with Sylsens B, applied at 8, 24, 48 and 72 h after inoculation of a microconidia suspension to human SC in the *ex vivo* model<sup>a</sup>

PDT application: time point, after spore inoculation (h)	Percentage fungicidal effect <sup>b</sup>			
	DMEM		distilled water	
	80 µM Sylsens B	160 µM Sylsens B	80 µM Sylsens B	160 µM Sylsens B
8	100	100	100	100
24	50 (±0)	50 (±0)	100	100
48	0	0	81 (±6)	96 (±5)
72	0	0	19 (±7)	65 (±8)

The values given are the means of four different experiments (±SEM).

<sup>a</sup>Incubation was either in DMEM (pH 7.4) or in distilled water (pH 5.2) for 2 h, followed by an illumination period with red light (108 J/cm<sup>2</sup>).

<sup>b</sup>A fungicidal effect is defined as the absence of any detectable fungal growth at 8 days after spore inoculation.

inoculation. When applying PDT at 48 and 72 h after inoculation, the treatment resulted only in a delay of growth, and no fungicidal effect was observed. However, in contrast to the results obtained with Sylsens B, the change of DMEM to water resulted in decreased efficacy. Only a small percentage of fungicidal effects could be obtained at the 8 h time point and no fungicidal effect was present at 24 h after spore inoculation or later. Both the light and dark controls in DMEM developed 10–15 cfu at day 8. In the case of the water medium, the light controls contained 10–15 cfu at each time point and the dark controls 5–12 cfu.

#### *Sylsens B is localized on the fungal wall after dark treatment and inside the fungal hyphae after successful PDT*

The DIC and fluorescence microscopy images shown in Figure 3 were made at different locations in the same preparation. In this

**Table 2.** Occurrence of a fungicidal effect after PDT with DP mme, applied at 8, 24, 48 and 72 h after inoculation of a microconidia suspension to human SC in the *ex vivo* model<sup>a</sup>

PDT application: time point, after spore inoculation (h)	Percentage fungicidal effect <sup>b</sup>			
	DMEM		distilled water	
	80 µM DP mme	160 µM DP mme	80 µM DP mme	160 µM DP mme
8	100	100	6 (±6)	54 (±6)
24	47 (±3)	85 (±7)	0	0
48	0	0	0	0
72	0	0	0	0

The values given are the means of four different experiments (±SEM).

<sup>a</sup>Incubation was either in DMEM (pH 7.4) or in distilled water (pH 5.2) for 2 h, followed by an illumination period with red light (108 J/cm<sup>2</sup>).

<sup>b</sup>A fungicidal effect is defined as the absence of any detectable fungal growth at 8 days after spore inoculation.

way we could select the most representative examples for both microscopic techniques.

Microscopic preparations of *T. rubrum* in the *ex vivo* model were made for four different conditions. Firstly, *T. rubrum* was visualized at 72 h after spore inoculation (see Figure 3a and b). It can be seen from Figure 3(b) that under the selected fluorescence microscopic conditions the fungus showed a green coloured autofluorescence. The images shown in Figure 3 (c and d), were taken from *T. rubrum* directly after the treatment with 160 µM Sylsens B in the dark at 72 h after spore inoculation. Both the DIC (Figure 3c) and the fluorescence images (Figure 3d) show that Sylsens B, represented by the red colour in the DIC images and the red/orange fluorescence in the fluorescence images, is localized on the fungal wall (see arrow points and inlays). Figure 3(e) (DIC) and Figure 3(f) (fluorescence) show images of the fungus after unsuccessful PDT using 80 µM Sylsens B, applied 72 h after spore inoculation. Again the arrows point (see inlays as well) to the localization of Sylsens B on the fungal cell wall.

Figure 3 (g and h) shows images taken from *T. rubrum* after a successful PDT with 160 µM Sylsens B, applied at 72 h after spore inoculation, when a fungicidal effect could be detected. Interestingly, the fluorescence image shown in Figure 3(h), taken at this stage shows that the red fluorescence of Sylsens B is present inside the fungal hyphae. The arrows in both the DIC and fluorescence images from this stage point to fungal hyphae filled with Sylsens B.

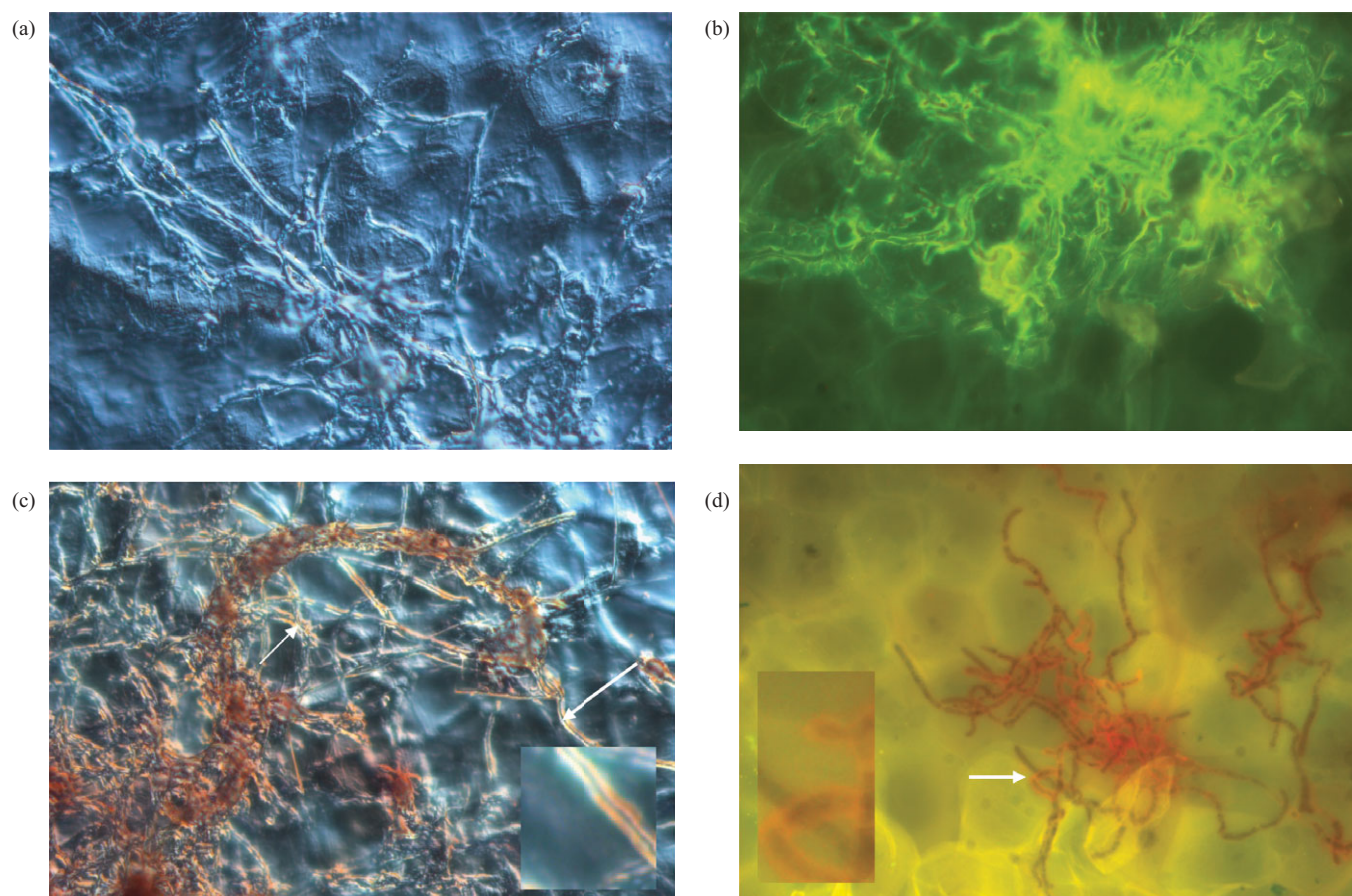
## Discussion

In this study we developed an *ex vivo* model in order to investigate the conditions and evaluate the efficacy of a PDT of the dermatophyte *T. rubrum*. Since our model offers the possibility of evaluating the therapeutic effect in different phases of the germination process, it may contribute to a better understanding of the way in which the therapy affects these individual growth phases. This is important since up to now only very few studies were concerned with the therapeutic fungicidal effects on different inoculates.<sup>15,16</sup> Recently, Seebacher<sup>7</sup> compared the efficiency of modern antifungal agents on both proliferating fungal strains and fungal spores.

Using our novel model, it has become clear that microconidia from *T. rubrum* attached to human SC can be completely inactivated by the porphyrin photosensitizer Sylsens B when PDT is applied at an early growth stage (8 h after spore inoculation). This result is in agreement with previous *in vitro* results where microconidia were used in suspension.<sup>13</sup> However, in these *in vitro* experiments, using the same light conditions, a much lower concentration (1 µM) of Sylsens B could be used for effective treatment. We suggest that the adherence of the microconidia to a substrate, like the SC in our *ex vivo* model, may influence the PDT susceptibility. This situation was different in our *in vitro* experiments when we worked with the suspensions and where the attachment to a surface did not play a role. Moreover, from our experiments it has become clear that the photosensitizer concentration is not the only factor playing a part in achieving a fungicidal effect. The occurrence of the fungicidal effect was strongly affected by the medium in which photosensitizers were applied. Using distilled water in combination with Sylsens B resulted in a high percentage of complete

fungal kill in every tested growth phase. From this observation we can also conclude that PDT can damage both the microconidia and fungal hyphae attached to SC. This is an important observation, since the pathogenesis is determined by the factors that control the fungal adherence, namely mechanical factors and production of the enzyme keratinase.<sup>14,17</sup> We propose that some local factors, adjusted by using water as an incubation medium, could be responsible for the remarkable difference in PDT efficacy. The pH of the water was 5.2, which was considerably lower than that of DMEM (pH 7.4). A lower pH may, in the case of Sylsens B, promote a selective binding to the fungus rather than to the SC. It is known that the isoelectric point of human skin is approaching pH 5,<sup>18</sup> which means that when an incubation pH of 5.2 is used the SC will be, at least partly, positively charged. Furthermore, we have found the first indications that the surface potential of both the fungal microconidia and hyphae is negative between pH 3.5 and 9.0. We therefore speculate that there will be a pH range where it is possible to promote a selective binding of the cationic photosensitizer, Sylsens B, to the fungus rather than to the skin. This reasoning can also

explain the result we obtained with DP mme. This porphyrin is negatively charged at both pH 7.4 and 5.2. However, in the latter case there may also be a substantial amount of D mme present that contains no net molecular charge due to the protonation of the carboxylic acid group (R. N. van der Haas, personal communication). We found that when using DP mme and DMEM the results resembled those obtained with Sylsens B. However, in the case of DP mme, changing the incubation medium from DMEM to distilled water did not improve the efficacy but decreased it instead. This result can be explained as follows: when incubating at pH 5.2 there will be a selective binding of the DP mme part that is negatively charged to the SC rather than to the fungus because of the positive charges present in the SC. This will, of course, decrease the efficacy of the treatment. The neutral part of DP mme will display an increased affinity towards the hydrophobic SC since the molecule has a more lipophilic character due to the protonated carboxylic acid group. It is also of importance that the aggregation of DP mme will increase at a pH of 5.2. Because of the complexity of the factors that may be of importance in the PDT effectiveness, we are



**Figure 3.** *Trichophyton rubrum* on human stratum corneum (SC) in the *ex vivo* model visualized with differential interference contrast (DIC) and fluorescence microscopy. Different locations in the same preparation were observed. *T. rubrum* on human SC at 72 h after spore inoculation (a: DIC, 400× and b: fluorescence, 400×). *T. rubrum* on human SC directly after treatment with 160 µM Sylsens B in the dark at 7 h after spore inoculation (c: DIC, 400× and d: fluorescence, 400×). *T. rubrum* on human SC after an unsuccessful PDT with 80 µM Sylsens B, applied at 72 h after spore inoculation (e: DIC, 400× and f: fluorescence, 400×). *T. rubrum* on human SC after a successful PDT with 160 µM Sylsens B, applied at 72 h after spore inoculation (g: DIC, 400× and h: fluorescence, 630×). The arrows point to the localization of Sylsens B (see also the inlays).



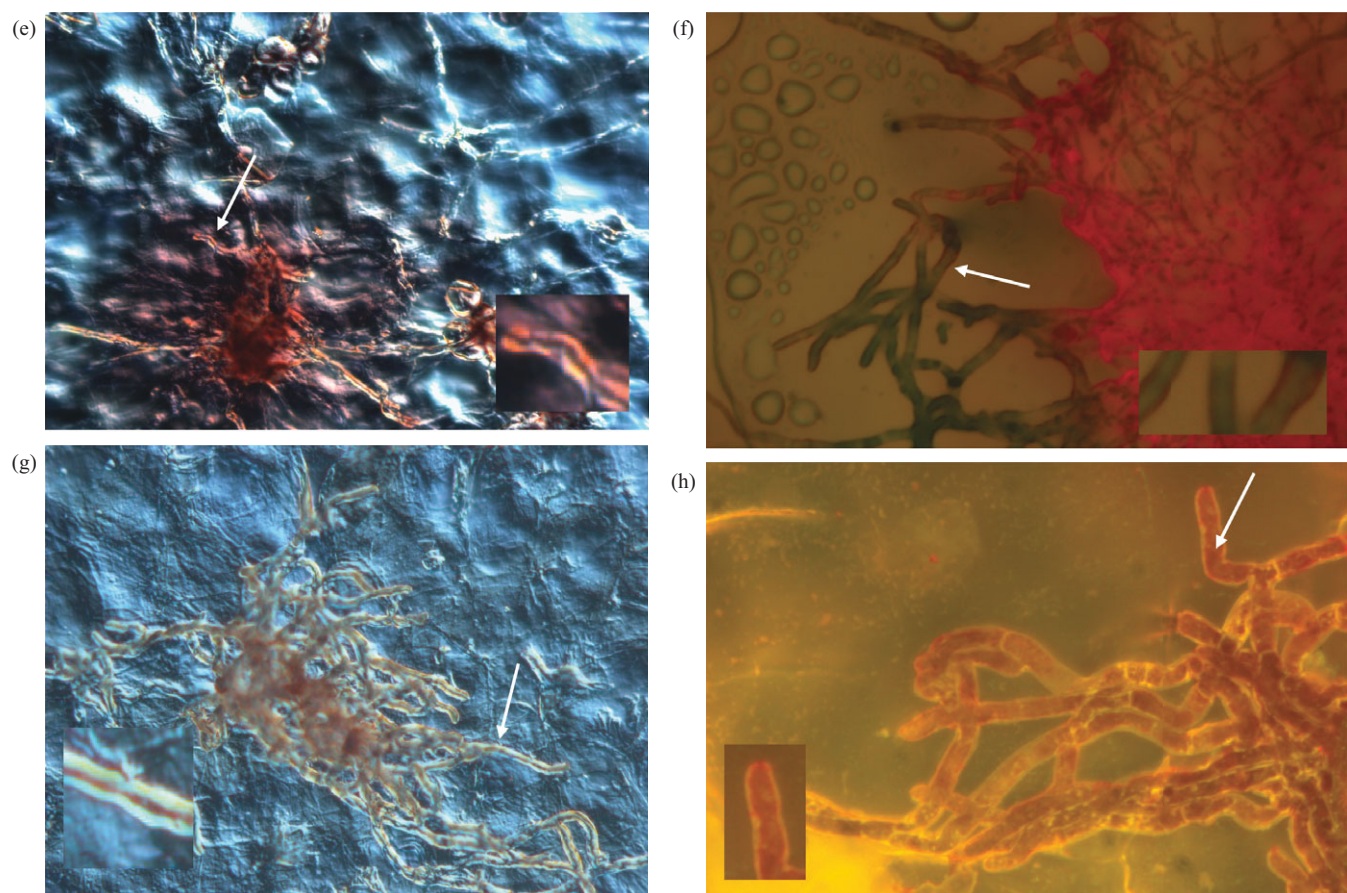


Figure 3. Continued

currently focusing on the mechanisms playing a role in the interactions between the photosensitizers and the surface of the targeted fungi. This investigation concerns research into the factors that can be of importance, like the ionic strength and pH of the surrounding medium, the surface potential of both microconidia and fungal hyphae, and the photophysical and photochemical properties of both photosensitizers.

The fluorescence and DIC images made from the different stages clearly illustrate that Sylsens B is localized on the fungal wall in the case of the dark controls and unsuccessful light treatments (Figure 3c, d, e and f). However, Figure 3 (g and f) shows a localization of the photosensitizer inside the fungal wall. Since the fungus was successfully treated with Sylsens B, a disruption of the fungal wall after PDT could be responsible for the penetration of Sylsens B inside the fungus. It can be concluded that in a situation that mimics the clinical situation, as represented by our *ex vivo* model, *T. rubrum* (both fungal spores and hyphae) can be successfully treated with PDT using 160  $\mu\text{M}$  Sylsens B and a light dose of 108  $\text{J}/\text{cm}^2$  (red light). The susceptibility of *T. rubrum* to PDT is clearly dependent on the time at which the treatment is performed. Application of the PDT at an increasing time after spore inoculation seems to decrease the susceptibility of the fungus to the treatment. This observation is in contrast with the experiences with regular antifungal therapeutics.<sup>19</sup> It should be emphasized also that the inactivation of the spores by PDT under the given circumstances

seems to be successful. This in contrast to many current therapeutic treatments where the spores are especially difficult to treat.<sup>7,8</sup> These non-responsive spores may remain in the skin where they can initiate a relapse of the infection. Our current research focuses on improving the PDT efficacy in the phases where fungal hyphae are treated. One may expect that the factors that influence the attachment of photosensitizer to fungal wall will be of essential importance in these growth phases. Also, the factors involved in the attachment of the fungus to the SC may be of importance. Controlling these factors is an essential condition for reaching maximal fungicidal effect.

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## Transparency declarations

None to declare.

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