The susceptibility of trichophyton rubrum to photodynamic treatment

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Chapter III

PHOTODYNAMIC TREATMENT OF THE DERMATOPHYTE *TRICHOPHYTON RUBRUM* AND ITS MICROCONIDIA WITH PORPHYRIN PHOTOSENSITIZERS

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Trichophyton rubrum microconidia, 17 hours after inoculation on human stratum corneum

ABSTRACT

The application of photosensitizers for the treatment of fungal infections is a new and promising development within the field of PDT. Superficial mycoses are probably the most prevalent of infectious diseases in all parts of the world. One of the most important restrictions of the current therapeutic options is the return of the infection and the duration of the treatment. This is especially true in the case of infections of the nail (tinea unguium) caused by *Trichophyton rubrum,* an anthropophilic dermatophyte with a worldwide distribution. Recently, we demonstrated that Sylsens B and DP mme were excellent photosensitizers toward *T. rubrum* when using broadband white light. This study demonstrates the photodynamic activity of these photosensitizers with red light toward both a suspension culture of *T. rubrum* and its isolated microconidia. The higher penetration depth of red light is important for the PDT of nail infections. In addition, we tested the photodynamic activity of a newly synthesized porphyrin, quinolino-[4,5,6,7-efg]-7-demethyl-8 deethylmesoporphyrin dimethylester, displaying a distinct peak in the red part of the spectrum. However, its photodynamic activity with red light toward a suspension culture of *T. rubrum* appeared to be only fungistatic. Sylsens B was the best photosensitizer toward both *T. rubrum* and its microconidia. A complete inactivation of the fungal spores and destruction of the fungal hyphae was found. In studies into the photostability, Sylsens B appeared to be photostable under the conditions used for fungal PDT. A promising result of this study is the demonstration of the complete degradation of the fungal hyphae in the time after the PDT and the inactivation of fungal spores, both with red light. These results offer the ingredients for a future treatment of fungal infections, including those of the nail.

INTRODUCTION

On irradiation with light of a proper wavelength, photosensitizers can initiate a photochemical reaction resulting in the production of $^1O_2$, which can react with cellular components. The sequence of events is termed photodynamic effect and can result in tissue destruction (1,2).

The application of photosensitizers for the treatment of fungal infections is within the field of PDT new and very promising.

Recently we demonstrated the susceptibility of the dermatophyte *Trichophyton rubrum* for photosensitizer-induced kill (3). *T. rubrum* is an anthropophilic dermatophyte with a worldwide distribution (4). Fungal infections of the skin are among the most common infections in humans. In the United States, 10% of the population has cutaneous fungal infections at any given time, and at least 40% may have it at sometime during life (5). One of the major limitations of the current therapeutic treatments is the return of the infection and the duration of the treatment (6,7). Topical treatment is satisfactory for isolated, mild type of lesions. In the case of widespread, more inflammatory or persistent dermatophytoses, systemic treatment is necessary. However, when using systemic therapy, one should be aware of potential risks of drug interactions and adverse effects like hepatotoxicity or general drug reaction, which can be of a serious nature. Topical antifungal agents are far less likely to cause adverse effects. A shortcoming of many antifungals is that they are only fungistatic (not fungicidal) and that is one of the reasons why they have to be applied during a long period of time. Because of the therapeutic weakness, there is still an urgent need for an effective and single treatment of tinea. This is especially true for the treatment of onychomycosis, fungal infection of nails, because this usually requires several months of systemic treatment (8,9).

We recently demonstrated using broadband white light that the porphyrins Sylsens B and DP mme were excellent photosensitizers to inactivate the dermatophyte *T. rubrum* (3). The effect we found for both Sylsens B and DP mme was a fungicidal one and not merely fungistatic. The efficacy of Sylsens B was found to be slightly higher than that found for DP mme. Structures formulae and related absorbance spectra are depicted in Fig. 3.1.

The aim of this study is to investigate the photodynamic efficacy of Sylsens B and DP mme on *T. rubrum* cultivated in suspension culture and on its microconidia using red light. The results thus obtained were compared with each other and with the results

obtained previously using a suspension culture of *T. rubrum* and white light only (3). Furthermore, the photostability of the porphyrin photosensitizers under study was evaluated.

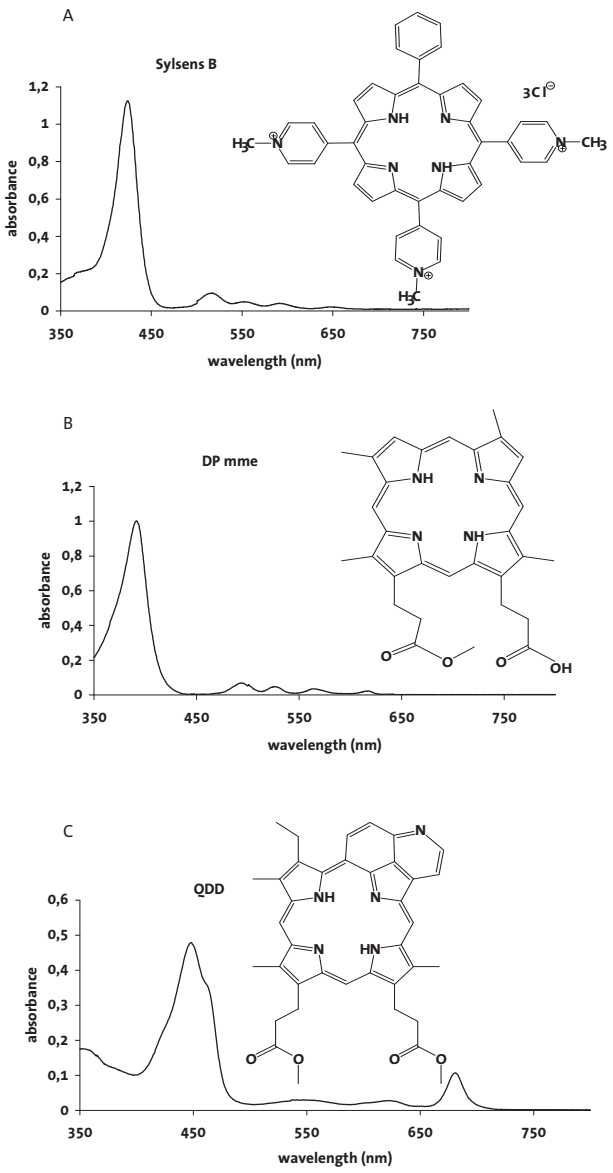

Figure 3.1. Absorbance spectrum in methanol and chemical structure of the porphyrin photosensitizers Sylsens B (A), DP mme (B) and QDD (C). The final concentration in all cases is 5 µM.

The possibility of the use of the red part of the spectrum for the PDT of tinea infections is of importance because red light has a considerably higher penetration depth in tissue than white light. This property can be particularly useful in the treatment of nail infections, which are among all tinea infections the most persistent to the current therapeutic treatments. Because of the importance of this topic, we investigated the efficacy of a newly synthesized porphyrin, quinolino-[4,5,6,7-efg]-7-demethyl-8-deethylmesoporphyrin dimethylester (QDD). This porphyrin has a distinct absorption peak in the red part of the spectrum (677 nm) and we therefore used it to establish the photodynamic activity with red light toward a suspension culture of *T. rubrum*.
Investigating the susceptibility of the microconia formed by *T. rubrum* is an important issue because they are, besides other types of spores, found in clinical situations. The studied topics will make a contribution to a future application of porphyrin photosensitizers in the PDT of tinea infections and in particular the infections of the nails.

MATERIALS AND METHODS

Materials

The fungus *T. rubrum* was purchased from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Cultures were grown on malt extract agar (MEA) (Oxoid, Hampshire, UK). For the preparation of a microconidia spore suspension, *T. rubrum* cultures were grown on Sabouraud dextrose agar (Sigma-Aldrich Chemie, Schnelldorf, Germany).
Suspension cultures were made in Dulbecco modified Eagle medium (DMEM, Gibco-BRL, UK) with 2.5% fetal calf serum (Gibco-BRL, Paisley, UK). Sylsens B, DP mme and QDD were synthesized and kindly provided by the Department of Bio-Organic Photochemistry, Leiden University, The Netherlands (purity, checked with nuclear magnetic resonance, was more than 99.5%).
Polyethyleneglycol was obtained from Genfarma B.V. (Maarssen, The Netherlands), whereas 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, polyethyleneglycol-ethanol-water (3:2:5) for DP mme and dimethylformamide (DMF) for QDD. Stock solutions of the photosensitizers of 0.7 mM were stored in solvent at 4°C for no longer than 1 week.

Preparation of rnicroconidia suspension

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 (10) with modifications, the use of a 0.01% Tween-80 solution in sterile water instead of phosphate-buffered saline and the use of a different filter (7 µm Millipore filter instead of a 8 µm Nucleopore filter).

On 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 µm diameter filter (Millipore, Molsheim, France). The effluent was centrifuged at 3400 *g* (IO°C), the resulting pellet washed with sterile water and suspended again in sterile water in a total volume of 2-4 mL (15 X 10^5 colony forming units [cfu]/mL). The obtained microconidia suspensions were stored in liquid nitrogen for no longer than 6 months. Counting the number of cfu on MEA dishes was used as a viability check. Identification of the isolated spores as microconidia was performed by the CBS in Utrecht, The Netherlands.

Light source

Illuminations were performed with a lamp from "MASSIVE" (no. 74900/21), 1 X 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 IL1400A photometer equipped with a SELO33/F/U detector (International Light, Newburyport, MA). A red cut-off filter at 600 nm was used to obtain the red part of the spectrum of the light produced by the lamp. The amount of UVA and UVB in the white light produced by the lamp was measured with a UVX radiometer (UVP, Upland, CA). The amount of UVA was less than 0.1% and the amount of UVB less than 0.007%.

Photodynamic treatment

PDT was applied to either a suspension culture of *T. rubrum* in DMEM or a spore solution in water. In all cases, illumination time was 1 h using a light irradiance of 30 mW/cm^2.

Before illumination, the fungal cultures in suspension were incubated with the photosensitizer in test tubes for 30 min at a temperature of 28°C. After incubation, the suspension cultures were illuminated in the presence of the sensitizer in 3 cm diameter culture dishes (Greiner, Alphen aan den Rijn, The Netherlands). After illumination, the contents of the culture dishes were transferred to 9 cm diameter

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dishes containing MEA, placed in the incubator at 28°C and growth was followed during 1 week and quantified by counting the number of inoculates present. The PDT of the microconidia was performed as described above but with an incubation time of 1 h at a temperature of 28°C.

Photostability

To determine the photostability of the photosensitizers within the time used for PDT, the spectral changes in absorbance (Shimadzu, UV-mini 1240's-hertogenbosch, The Netherlands) and emission (Perkin-Elmer LS 50B luminescence spectrometer, Beaconsfield, Buckinghamshire, England) were measured in Dulbecco phosphate-buffered saline (DPBS) during a period of 0, 30 and 60 min. of illumination.

RESULTS

PDT of T. rubrum in suspension culture

To compare the results with the previously obtained results with white light (3), we used a concentration range up to *5* µM of photosensitizer. Using white light, the PDT-effective concentration range was for both Sylsens B and DP mme below 5 µM (4 µM, 3 µg/mL, for Sylsens B and 5 µM, 3 µg/mL for DP mme). To establish a true fungicidal effect using red light, we expanded the concentration range up to 50 µM in additional experiments.

Using red light and a suspension culture of *T. rubrum,* Sylsens B displayed high photodynamic activity. As can be seen from Fig. 3.2A and B, a concentration of 5-10 µM resulted in complete kill (on the basis of a cutoff at two colonies). On comparing this result with the result obtained for DP mme under the same experimental conditions, it can be seen (Fig. 3.2C,D) that for DP mme a true fungicidal effect could not be obtained below a concentration of 40 µM. Below this concentration, PDT only results in a delay in growth.

The result obtained for QDD however was a fungistatic one (Fig. 3.2E). Seven days after PDT the number of fungal spots present on the agar dishes was equal to the number of spots on the control dishes. Below 10 µM, no effect at all could be established for this porphyrin photosensitizer. However, on using Sylsens B and DP mme in the same concentration range, complete fungal kill could be observed.

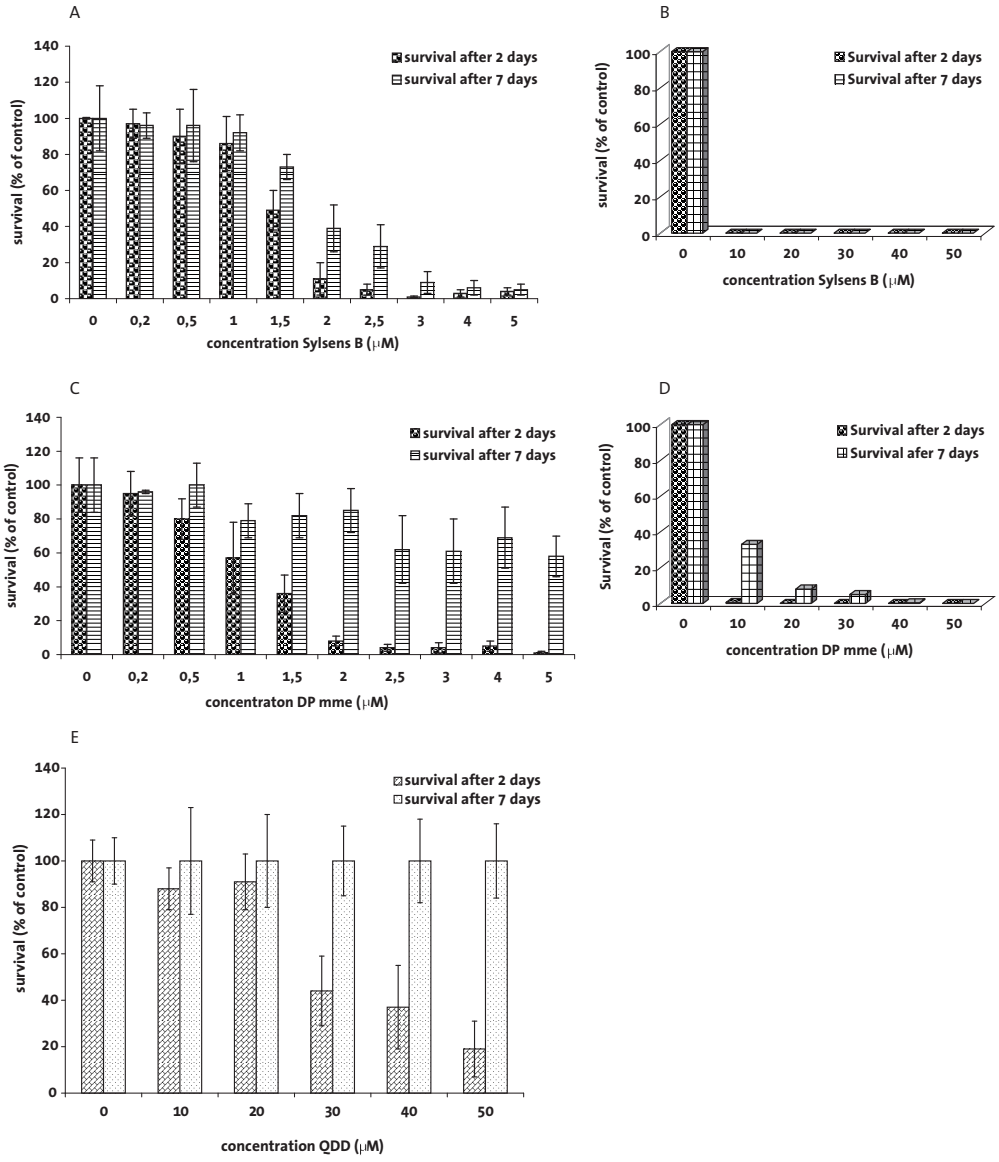

Figure 3.2. Growth of *T. rubrum* after PDT with Sylsens B (A,B), DP mme (C,D) and QDD (E) using red light. Suspension cultures were incubated in the dark for 30 min at 28°C with different concentrations of Sylsens B, DP mme or QDD. After the incubation period, suspension cultures were illuminated with red light (108 J/cm^2) in the presence of the photosensitizer. After illumination, cultures were transferred to MEA dishes and placed in the incubator at 28°C. The survival was measured after 2 and 7 days as the number of inoculates present. Untreated cultures containing solvent instead of photosensitizer were considered as 100% survival. Number of inoculates on the control dishes: 200-240 after 2 days and 380-450 after 7 days. The values given are the means of three experiments with the standard deviation. For the result presented in Fig. 3.2B, the values for the standard deviation are as follows: 100 ± 15% for the survival after 2 days and 100 ± 11% for the survival after 7 days. For the result presented in Fig. 3.2D, the values for the standard deviation are as follows: 100 ± 18% for the survival of the control after 2 days and for the survival after 7 days, 33 ± 25% for 10 μM, 8 ± 2% for 20 μM, 5 ± 3% for 30 μM and 100 ± 12% for the control.

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For every photosensitizer, appropriate dark-control experiments were included, resulting in no dark toxicity for the used porphyrin photosensitizers under the given circumstances.

For the porphyrin compounds Sylsens B and DP mme, it was established that after successful PDT no reoccurrence of the fungus on the MEA dishes was observed even after several weeks. Microscopically however we discovered that on these successfully treated cultures small hypha fragments could still be detected on the MEA dishes up to at least 2-3 weeks after PDT. However, as shown in Fig. 3.3, they were no longer viable and degraded further into very small fragments.

PDT of T. rubrum microconidia

Using microconidia isolated from *T. rubrum* we investigated the susceptibility of these spores for PDT. Only the porphyrin photosensitizers that were proven to give a true fungicidal effect toward *T. rubrum,* Sylsens B and DP mme, were used.

To establish a photodynamic effect, the viability of the spores was checked by their ability to form cfu after PDT when subcultured on MEA. The number of cfu thus obtained was compared with the number obtained in the control dishes containing spore suspensions treated with light and solvent instead of photosensitizer. Preliminary experiments varying the incubation time at 28°C and the photosensitizer concentration up to 50 μM showed that a 1 h incubation time was sufficient to establish a distinct PDT effect using 108 J/cm^2 of red light. At the lowest concentration used in these experiments (10 μM for Sylsens B and DP mme), the microconidia were unable to geminate at a temperature of 28°C. The result thus obtained was checked every week, up to a final period of 3 months. During that time no germination could be detected. On the basis of the results of these preliminary experiments, the photodynamic efficacy of Sylsens B and DP mme toward microconidia isolated from *T. rubrum* was tested in a concentration range up to 5 μM. As can be seen from Fig. 3.4, there is a high photodynamic efficacy of both photosensitizers towards microconidia. Using Sylsens B the germination of the microconidia was completely inhibited (Fig. 3.4A). However, the efficacy with red light was found to be quite high. Complete inhibition already occurred at a concentration of 1 μM. The result for DP mme (Fig. 3.4B) was somewhat different. Although also displaying a high photodynamic efficacy, a complete inhibition could only be detected at concentrations higher than 5 μM.

In all cases the effect of PDT was followed for a period of 3 months. It was found that in all cases where a complete inhibition was observed in the first week after PDT, this

result remained unchanged during the follow-up period of 3 months.
For both Sylsens B and DP mme, appropriate dark-control experiments were included, resulting in no inhibition in spore germination under the given circumstances. However, we did observe a delay in germination time because of the light submitted to the spore suspension culture.

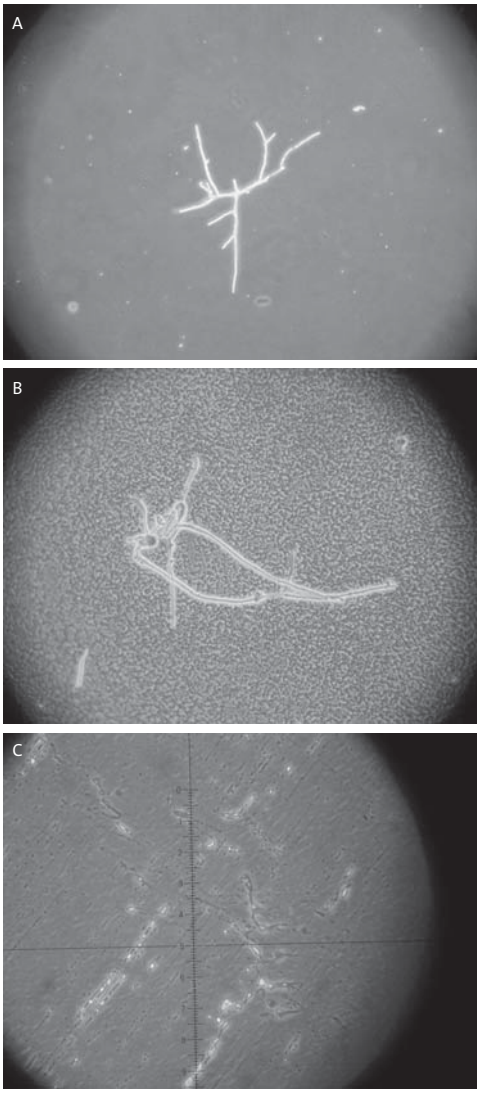

Figure 3.3. Picture taken (Minolta Dimage xi digital camera) from a microscope image (Zeiss Axiovert 25, with 320X magnification) showing hyphen 2.5 weeks after successful PDT with Sylsens B (A) compared with an image of viable hyphen (B) of an untreated culture starting to grow. After 3.5 weeks, the fragmentation of the hyphen is clearly visible (C).

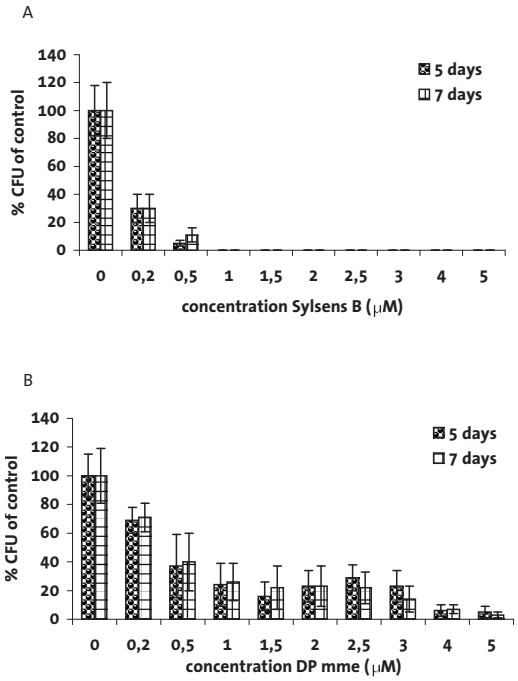

Figure 3.4. Photodynamic effect of Sylsens B (A) and DP mme (B) on the ability of forming cfu from *T. rubrum* microconida using red light. Microconidia suspensions were incubated in the dark for 1 h at 28°C with different concentrations of Sylsens B or DP mme. After the incubation period, spore suspensions were illuminated with red light (108 J/cm^2) in the presence of the photosensitizer. After illumination, spore suspensions were transferred to MEA dishes and placed in the incubator at 28°C. The number of cfu was determined after 5 and 7 days. The number of cfu from untreated spore suspensions containing solvent instead of photosensitizer was considered as 100%. Number of cfu on the control dishes: 70-100. The values given are the means of three experiments with the standard deviation.

Photostability

To get an impression of the photostability of the porphyrin sensitizers under study, we investigated the spectral changes in absorbance in the visible part of the spectrum and the changes in emission before and after illumination with broadband white light. All the spectra were taken in DPBS after 0, 30 and 60 min of 30 mW/cm^2 (0, 54 and 108 J/cm^2).

For QDD the observed spectral changes in absorption are shown in Fig. 3.5A, and the changes in the fluorescence emission spectrum for this compound can be seen in Fig. 3.5B. A decrease in the Soret peak was observed of more than 30% under the given experimental illumination conditions. The result obtained for the emission spectrum is comparable.

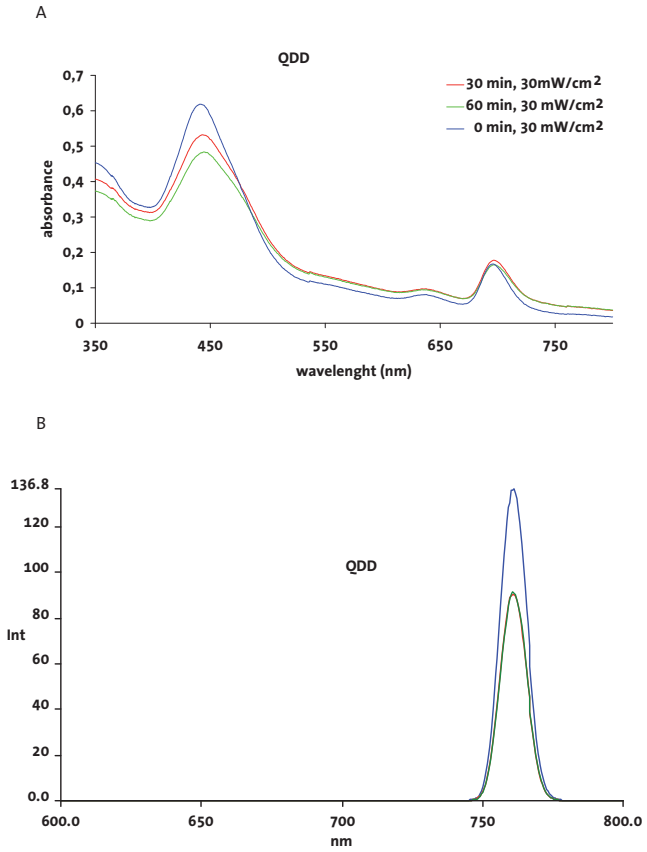

Figure 3.5. Absorption (A) and emission (B) spectra of QDD taken in DPBS before and after illumination with broadband white light (30 mW/cm^2). The excitation wavelength was 450 nm and the final concentration of QDD was 15 µM. Blue, 0 min illumination; red, 30 min illumination, 54 J/cm^2; green, 60 min illumination, 108 J/cm^2.

For Sylsens B the absorption spectrum taken in DPBS resembled the corresponding spectrum in methanol. Furthermore, no spectral changes could be observed on illumination of this photosensitizer (data not shown).

The absorbance spectrum for DP mme in DPBS differs slightly from the spectrum taken in methanol, but we did not observe any substantial additional changes on illumination (data not shown).

No spectral changes were observed for Sylsens B and DP mme under the illumination conditions used in the PDT experiments, whereas for QDD a decrease in peak height was observed, indicating substantial bleaching.

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DISCUSSION

Using red light the porphyrins Sylsens B and DP mme were both proven to be effective photosensitizers for the inactivation of *T. rubrum* cultivated in a suspension culture. Sylsens B was found to be the most effective. Using red light a complete fungicidal effect was obtained at a relatively low concentration. Previous results obtained with white light showed that using a light dose of 108 J/cm^2 and a concentration of 4 µM of Sylsens B, a complete kill of *T. rubrum* could be obtained (3). Using the same dose of red light, about twice as much of the sensitizer has to be used to obtain a comparable fungicidal effect. Using DP mme in combination with red light, a much higher concentration of 40 µM was necessary to obtain an equivalent fungicidal effect. The slightly higher absorbance in the red part of the spectrum by Sylsens B compared with DP mme could account for the significant difference in photodynamic effect. The result obtained for QDD cannot be explained in the same way because this compound is actually the only porphyrin photosensitizer tested here that has a large absorption peak in the red part of the spectrum. The observed difference in photodynamic efficacy between the sensitizers cannot be explained by differences in $^1O_2$ quantum yield. The quantum yield (11) of the three compounds was of the same order of magnitude.

We speculate that the positive charges present in the Sylsens B molecule could account, at least partly, for its success in PDT toward this fungal system. There might be binding to the fungal wall. The hydrophilic properties of Sylsens B will enable and facilitate this process. For the less hydrophilic and under biological conditions negatively charged porphyrin DP mme there might occur binding because of the fact that amino acids such as lysine and histidine are present in the fungal mycelium.

However, the porphyrin QDD is rather hydrophobic, a property that might complicate the approach and subsequent binding to the fungal colonies present in the suspension culture. We furthermore observed a substantial decrease in fluorescence for QDD in DMF in the presence of an increasing percentage of water (data not shown). This observation indicates aggregation of the photosensitizer, leading to a decrease in photodynamic efficacy in a medium containing water. This is consistent with the fact that we observed that most of the very hydrophobic porphyrin photosensitizers tested in our laboratory did not display any photodynamic effect toward the fungal system (G.M.T. Smijs, unpublished). The lack of a positive or negative charge in the QDD molecule could also complicate binding to the fungus.

Investigating the PDT susceptibility of the microconidia produced by *T. rubrum,* we

found that both porphyrins Sylsens B and DP mme were effective photosensitizers. We again found that Sylsens B induced complete kill at lower sensitizer concentrations. A concentration of 1 μM was sufficient to prevent germination.

PDT-treated microconidia were followed for a period of more than 3 months. Germination was never observed. In the case of DP mme, more than 5 μM was necessary to establish the same effect.

In conclusion it can be said that the porphyrin photosensitizer Sylsens B is in every aspect the best photosensitizer to treat both the suspension culture and the microconidia of *T. rubrum*; compared with suspension culture, the efficacy toward the microconidia was found to be higher. Important in this aspect is the fact that this photosensitizer shows an excellent photodynamic efficacy with red light, a property that will be convenient in our search for a photosensitizer, which may be used for the treatment of tinea infections of the nail. In this aspect 10 μM of Sylsens B is considered to be a safe concentration.

Because of the expected increased tissue penetration depth of red light, we synthesized and tested the compound QDD, displaying a distinct absorption band in the red part of the spectrum. However, this compound was not effective under the experimental conditions used. In the future, it could be worthwhile to modify the water solubility while maintaining its spectral properties.

The most important conclusion to draw from this study is the fact that PDT causes complete destruction of the fungal hyphae, an effect that was confirmed by the CBS, Utrecht, The Netherlands (R. C. Summerbell, personal communication). The fact that *T. rubrum* spores can also be inactivated using PDT will bring us closer to the development of a PDT protocol for treating tinea infections, In a clinical situation the spores are responsible for the initiation of a tinea infection and the same spores will frequently remain and survive on the skin after medical treatment, enabling the start of a new infection. In future experiments, we will explore the mechanism behind the photodynamic effect demonstrated here to come to a better understanding of what may offer a promising worldwide application for PDT.

ACKNOWLEDGEMENTS

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