The susceptibility of trichophyton rubrum to photodynamic treatment

Smijs, G.M.T

Chapter V

INVESTIGATION OF CONDITIONS INVOLVED IN THE SUSCEPTIBILITY OF THE DERMATOPHYTE *TRICHOPHYTON RUBRUM* TO PHOTODYNAMIC TREATMENT

Threes G.M. Smijs[1], Joke A. Bouwstra[2], Mojgan Talebi[1] and Stan Pavel[1]

[1] **Leiden University Medical Centre**, Leiden, The Netherlands.

[2] **University of Leiden**, Leiden/Amsterdam Centre for Drugs Research, Leiden, The Netherlands.

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Trichophyton rubrum mycelium (8 days after microconidia inoculation)

ABSTRACT

PDT refers to a treatment with light-activated agents (photosensitizers) in combination with visible light and molecular oxygen. Recently, we have demonstrated that the porphyrins, Sylsens B and DP mme are excellent photosensitizers to be used against *Trichophyton rubrum* both *in vitro* and *ex vivo*.

The objective of this study was to investigate the key factors involved in PDT efficacy of both photosensitizers in an *ex vivo* situation during different fungal growth stages using a recently developed *ex vivo* model. The study focused on the influence of pH and ion strength of incubation media, photochemical properties of the photosensitizers (spectra and singlet oxygen production), and the effect of several scavengers of reactive oxygen species (sodium azide, histidine, mannitol) and phenylmethylsulphonylfluoride (keratinase inhibitor) on the PDT efficacy.

The results show that an optimal pH and low concentrations of calcium are crucial for a selective binding of Sylsens B to the fungus, leading to an increased PDT efficacy. This selective binding to *T. rubrum* cannot be accomplished for DP mme. It can be concluded that the prerequisite for successful treatment is the use of a low molarity solution of pH 5, supplemented with a chelating agent and a keratinase activity-repressing agent. Under these conditions, PDT with Sylsens B inactivates, initially via singlet oxygen, effectively the fungus in different fungal growth stages.

INTRODUCTION

Upon irradiation with light of an appropriate wavelength and in the presence of molecular oxygen, photosensitizers can initiate a photochemical *type I* or a *type II* reaction or a combination of both (1,2). In a *type I* reaction, the activated photosensitizer reacts with a substrate molecule by either an electron or a hydrogen transfer, leading to the formation of radicals. In a *type II* reaction, an energy transfer occurs to the ground state of molecular oxygen, leading to the production of the reactive $^1O_2$. As a consequence of both pathways, the photodynamic effect can result not only in selective tissue injury, but also in the elimination of different kind of pathogens if they are present in the direct neighbourhood of the photosensitizer (3). Because of the short lifetime of singlet oxygen ($^1O_2$), a selective binding of the photosensitizer to the target organism is a precondition of high PDT effectiveness (3,4). In the presence of a high oxygen concentration, however, the energy transfer, leading to the production of $^1O_2$, is favoured (5). It is generally agreed that $^1O_2$ is the key agent responsible for the cellular damage during PDT (6-8). The application of PDT for fungal infections is a new and promising avenue within the field of PDT (9-11). The dermatophyte *Trichophyton rubrum* is the most important cause of tinea (12,13) and the infections caused by this dermatophyte can be very persistent (14). The most important limitations of the current therapeutic treatments for tinea are the recurrence of the infection and the duration of the treatment (15). New treatment strategies like PDT can offer a solution to this problem. Recently, we have demonstrated that the porphyrins, Sylsens B and DP mme are excellent photosensitizers towards *T. rubrum*. Both photosensitizers have a fungicidal effect (a complete inactivation of both fungal spores and hyphae) within one single PDT, both in an *in vitro* (16,17) and *ex vivo* situation (18). The fungicidal effect of Sylsens B could be increased when distilled water was used instead of Dulbecco's modified Eagle's medium (DMEM) as an incubation medium. However, in case of DP mme, changing the incubation medium from DMEM to water reduced the PDT efficacy (18).

The aim of this study was to unravel the mechanisms involved in PDT efficacy of both photosensitizers towards *T. rubrum*. First, we focused on two factors (pH and ion strength of the incubation media) that could be responsible for the remarkable difference in PDT efficacy of these photosensitizers. For this purpose, we used our recently developed *ex vivo* model (18). We investigated the influence of above-mentioned factors on the PDT efficacy on three different fungal growth stages

that were present 17, 48 and 72 h after spore inoculation on the stratum corneum (SC). Under the given circumstances, the 17 h growth stage was characterized by germinating microconidia and the absence of fungal hyphae. At 48 h after spore inoculation, the microconidia germination was completed and fungal hyphae started to appear. The 72 h growth stage represented a complete hyphae stage.

In order to characterize the binding properties of the porphyrins with microconidia and hyphae from *T. rubrum*, the zeta potential was measured as a function of pH. We also evaluated the effect of Alcian Blue (AB) and Ca^{2+} ions as competitors for the binding of Sylsens B and DP mme to *T. rubrum*. Calcium ions are known to be natural constituents of the human skin environment and AB was used as a model compound that has been reported to bind to the negative-charged mannophosphate groups present on the outer fungal wall (19).

As the photosensitizing properties of the porphyrins (and therefore the efficiency) can be pH dependent, the light absorption, emission and singlet oxygen production were studied in relation to the pH. The photodynamic efficacy of the two photosensitizers was also tested in the presence of sodium azide, histidine, mannitol and phenylmethylsulphonylfluoride (PMSF). Sodium azide and histidine are known quenchers of $^1O_2$ (20),and mannitol binds hydroxyl radicals (21). PMSF is known to inhibit the keratinase activity produced by *T. rubrum* (22).The latter is thought to play a role in the process of pathogenesis following attachment of the dermatophyte to human skin (23,24).The results thus obtained can contribute to a better understanding of the differences in the susceptibility of the different growth phases of *T. rubrum* to a PDT. The optimization of the conditions for PDT of *T. rubrum* is also of importance for the development of PDT of other superficial mycotic skin infections.

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 (Sigma-Aldrich Chemie, Germany) at room temperature. For the preparation of a hyphae suspension, *T. rubrum* was cultivated at room temperature in a suspension culture using DMEM (GibcoBRL, UK) supplemented with 2.5% fetal calf serum (FCS; GibcoBRL).

The photosensitizer DP mme (mol. wt: 524.61 g/mol) was synthesized by the Department of Bio-Organic Photochemistry, Leiden University, The Netherlands (purity, determined with NMR was more than 99.5%) and kindly provided to us. The photosensitizer Sylsens B (mol. Wt: 769.16 g/mol) was synthesized by Buchem Holding BV (Lieren, The Netherlands) and the purity was 99% according to NMR measurements.

Trypsin, PMSF, sodium azide, tryptophan and AB were obtained from Sigma (Zwijndrecht, The Netherlands), Blankophor from Bayer Chemicals (Mijdrecht, the Netherlands), Triton X-100 from Fluka (Fluka Chemica, Switzerland), whereas all other chemicals were purchased from J. T. Baker (Deventer, The Netherlands).

Photosensitizer solutions were prepared in water with an adjusted indicated pH or a buffer solution of indicated pH and molarity. For pH values ranging from 3 to 5.2 a citric acid/sodium citrate buffer was used, for pH 7.4 a phosphate buffer and for pH 9 a tris(hydroxymethyl)aminomethane (Tris) buffer.

Preparation of microconidia suspension

The protocol to obtain a suspension of microconida produced by *T. rubrum* grown on Sabouraud Dextrose Agar was based on a method described previously (18,25).The obtained microconidia suspensions (10–40 x10^6 cfu/mL) were stored in liquid nitrogen for no longer than 6 months. Counting the number of cfu on malt extract agar (MEA) dishes was used as a viability check.

Preparation of hyphae suspension

From a 4- to 5-day-old *T. rubrum* suspension culture in DMEM, a volume of 25 mL was taken from beneath the liquid surface and centrifuged for 20 min at 10°C (3400 *g*). The pellet was washed twice with water and suspended in 4 mL of water. The obtained suspension was placed in an ultrasonic water bath for ~15 min and subsequently stored at 4°C for no longer than 24 h. 0.5–1 mL of sample was taken and incubated for 2 h with an equal volume of Blankophor (1:10 diluted in water). After washing, the hyphae pellet was taken up in 0.5 mL of water. A few drops were placed on an object glass, covered with a coverslip and inspected microscopically (Leica CTR 5000) for microconidia contamination using a DAPI fluorescence filter (UV, PB420/30).

Preparation of the human 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 ~250 µm 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 PBS of pH 7.4 (4°C) overnight. After 1 h at 37°C, the SC was removed manually. The obtained SC was dried in the air for 24 h and kept under nitrogen over silica gel for no longer than 3 months.

Zeta potential

The zeta potential can be defined as the potential difference measured at the junction between a particle and its closely related counter ions and the bulk solution. In dilute solutions, the zeta potential is a measure for the surface potential of a particle (26,27). Zeta potential measurements (Zetasizer 2000/3000, Malvern Instruments Ltd, Worcs, UK) were performed on both microconidia and fungal hyphae dilutions in a 1 mM buffer solution of different pH (3–9.4). Measurements were performed on three different microconidia and hyphae isolates. For every isolate, three different measurements were performed for every indicated pH and every measurement contained 10 runs.

Absorption and emission spectra

Absorption (Shimadzu UV mini 1240, Den Bosch the Netherlands) and emission spectra (Perkin-Elmer LS 50B luminescence spectrometer, Perkin-Elmer Nederland BV) were taken from both Sylsens B (2.5 µM) and DP mme (10 µM) in a 5 mM buffer solution in the pH range 3–9 or in methanol.

Singlet oxygen production

Singlet oxygen production was measured indirectly by measuring the photo-oxidation of 4 mM tryptophan in the presence of 20 µM Sylsens B or DP mme in a 50 mM buffer solution of indicated pH (3.5, 5.2 or 7.4). Samples were illuminated (Philips projection lamp, type 7158X HP pcs A1/216) in a closed system (21–22°C) using a flux-rate of 21–22 mW/cm^2 and a red cut-off filter at 580 nm to obtain the red part of the spectrum. The oxygen consumption was measured using a YSI 5300 biological oxygen monitor (YSI Incorporated, Yellow Springs, OH, USA). The rate of oxygen consumption was calculated from the initial slope of the recorded curve and expressed as % O_2/min. All measurements were repeated three times for every pH.

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Binding assays

A binding assay for Sylsens B to the fungus at pH 5.2 and 7.4 in the absence and presence of either $CaCl_2$ (5 mM) or AB (160 µM) was performed. For DP mme, the binding assay was performed at pH 5.2 and 7.4 in the presence and absence of AB (160 µM). Two millilitres of a suspension culture of the fungus was centrifuged (4300 *g*) for 20 min and washed twice with 2 mL of water of pH 5.2 or 7.4. The pellet was then taken up in 2 mL of water of pH 5.2 or 7.4 supplemented with 160 µM Sylsens B or 200 µM DP mme. After an incubation period of 17 h, the fungus was centrifuged and the fluorescence present in the supernatant measured in methanol upon excitation at 424 nm for Sylsens B and 392 nm for DP mme (Perkin-Elmer LS 50B luminescence spectrometer, Perkin-Elmer Nederland BV). The influence of calcium ions and the cationic dye AB on the binding capacities of the photosensitizers to the fungus was investigated by incubating the fungus with 5 mM $CaCl_2$ (Sylsens B) and 160 µM AB (Sylsens B and DP mme) for 24 h prior to the photosensitizer incubation. Competition between Ca^{2+} ions and Sylsens B for the available fungal binding sites was investigated by a 17 h incubation of the fungus in suspension with both Sylsens B (160 µM) and $CaCl_2$ (5 mM). In addition, the binding of Sylsens B and DP mme to SC was tested at pH 5.2 and 7.4. Two millilitres of water (pH 5.2 or 7.4) containing human SC (2.4 cm^2) was incubated with 160 µM Sylsens B or 200 µM DP mme. After 17 h of incubation, the SC was removed, washed and the remaining fluorescence measured in methanol upon excitation at 424 nm for Sylsens B and 392 nm for DP mme.

Ex vivo model

The *ex vivo* model was used as described previously (18). A microconidia suspension was diluted to 1000 cfu/mL and 15 µL inoculated on the circular piece of human SC in the model. The MEA dish, containing the inoculated SC, was placed in an incubator at 28°C and at 17, 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 the appearance of fungal hyphae can be detected microscopically (Zeiss Axiovert 25) at 48 and 72 h after their inoculation.

Light source PDT

Illuminations were performed with a lamp from 'MASSIVE' (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 absorbing infrared light was used. Light intensity was measured with

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 part of the spectrum of the light produced by the lamp. The wavelength range of the emitted light was 580–870 nm and the light intensity at the level of the infected human SC was 30 mW/cm^2.

Photodynamic treatment

At 17, 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 µL of incubation medium. The incubation medium contained either 1 mL of distilled water with an adjusted pH (3.5, 5.2 or 7.4) and 35 µL of a photosensitizer solution (final concentration 5–200 µM) or 1 mL of a citric acid/sodium citrate buffer pH 5.2 of different molarity (5–500 mM) supplemented with the photosensitizer. In case of DP mme, incubation was in a 5 mM buffer solution of pH 5.2 (citric acid/sodium citrate) or pH 7.4 (sodium phosphate). To study the effect of sodium azide, histidine or mannitol on the PDT efficacy of the porphyrins at 17 h after spore inoculation, 2.5 mM sodium azide, 4 mM histidine or 10 mM mannitol was added to the incubation medium containing Sylsens B or DP mme prior to the irradiation period. The effect of PMSF on the PDT efficacy of Sylsens B was tested at 72 h after spore inoculation by addition of 1 mM PMSF to the incubation medium containing 40 µM Sylsens B. During the incubation period of 2 h (3 h in the presence of PMSF), the membrane filter with the SC was turned upside down. 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, to allow the surface of the SC to face the lamp. In all cases, the illumination time was 1 h using a light flux rate of 30 mW/cm^2, corresponding to a light dose of 108 J/cm^2. After the illumination, the membrane filter with the SC was transferred to a fresh MEA dish, placed in a 28°C incubator and fungal growth was followed for at least 8 days. 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 alone in the presence of light. The efficacy of the treatment was expressed as the relative frequency of complete inactivation of fungal growth detected at day 8 after spore inoculation, defined as a fungicidal effect. To assess this, a Zeiss Axiovert 25 microscope was used. If at day 8 by visual inspection, no regrowth could be observed a complete inactivation of spores and hyphae as a result of PDT was established.

Statistical analysis

Statistical analysis of the *ex vivo* results was performed using Friedman's ANOVA for related samples, followed (if necessary) by the Wilcoxon signed-rank test (SPSS 12.0.01). The critical level of significance used was 0.05 (*P* values given are two-tailed). In all other cases, the independent Student's *t* -test (*P* = 0.05) was applied.

RESULTS

Zeta potential of both microconidia and fungal hyphae is pH dependent

Fig. 5.1 shows the results obtained from the zeta potential measurements on microconidia and fungal hyphae in 1 mM buffer solutions of the indicated pH. In the tested pH range of 3–9.4, a clear pH dependence of the zeta potential could be observed for both the microconidia and hyphae. In addition, the results show that the iso-electric point (pI) of both the microconidia and fungal hyphae is between pH 3 and 3.5.

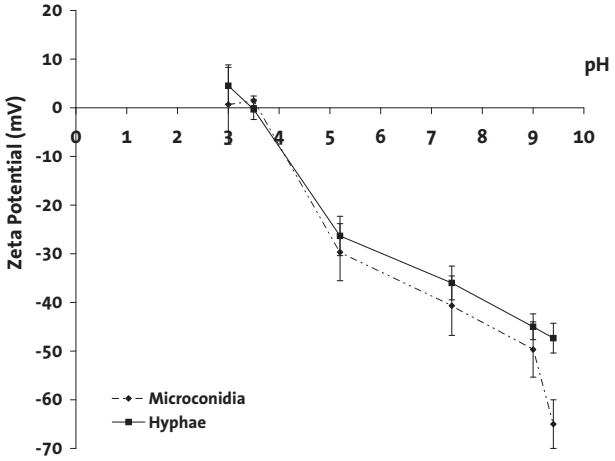

Figure 5.1. Zeta potential of *T. rubrum* microconidia and fungal hyphae, measured as a function of the pH. Values given are the mean values for measurements on three different microconidia and hyphae isolates including the SD.

Absorption and emission spectra of Sylsens B are not influenced by the pH, while changing the pH caused a difference in DP mme spectral behaviour

As can be seen from Fig. 5.2, changing pH did not influence the spectral behaviour of Sylsens B. Sylsens B was apparently present in an aggregated form in all the buffer solutions. This can be inferred from the characteristics of the emission spectra in Fig. 5.2B. To show the spectral behaviour of porphyrins in monomeric form, the absorption and emission spectra in methanol were also measured and included in the figures.

In case of DP mme, both the absorption (Fig. 5.3A) and fluorescence (Fig. 5.3B) were influenced by the pH. The light absorption at pH 3 and 4.5 was low and no fluorescence could be measured. Furthermore, a blue shift was detected for the Soret band wavelength from pH 9 to 3. The Soret band wavelength for pH 9 (390 nm) resembled the value found for the absorption spectrum in methanol (392 nm), but at pH 3 the Soret band wavelength shifted to a value as low as 351 nm.

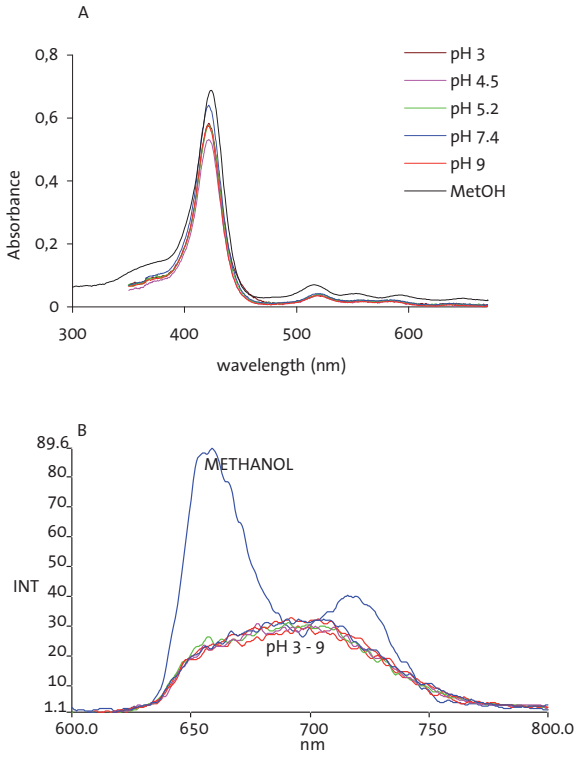

Figure 5.2. Absorption (A) and emission spectra (B) for Sylsens B (2.5 µM), taken in a 5 mM buffer solution of indicated pH or methanol. INT, fluorescence intensity expressed in arbitrary units.

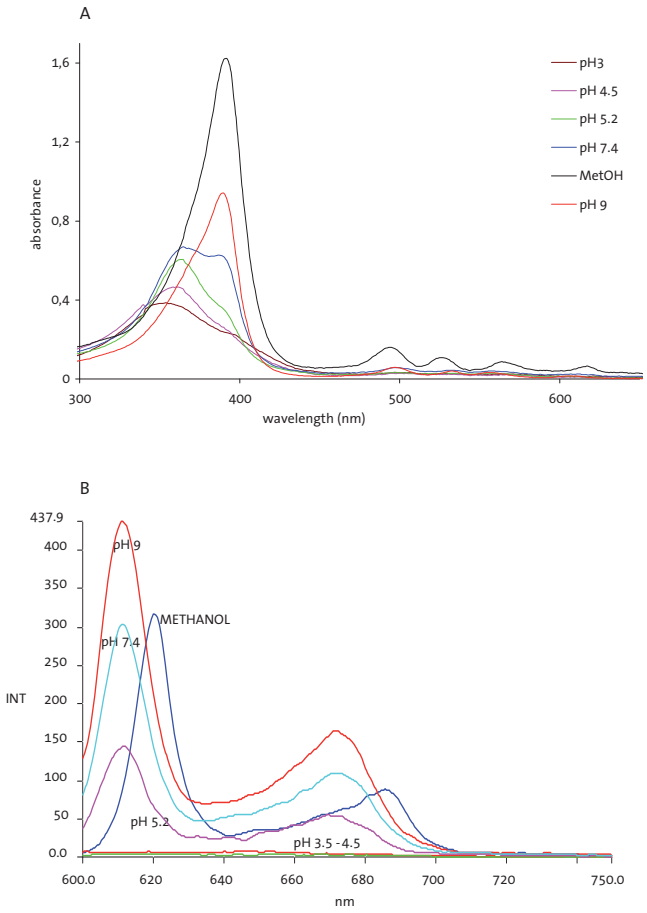

Figure 5.3. Absorption (A) and emission spectra (B) for DP mme (10 µM), taken in a 5 mM buffer solution of indicated pH or methanol. INT, fluorescence intensity expressed in arbitrary units.

pH influences only the singlet oxygen production of DP mme

The results of oxygen consumption as a measure for the singlet oxygen production are given in Table 5.1. We determined the quenching of singlet oxygen by using tryptophan since the photo-oxidation of tryptophan by singlet oxygen is not influenced by the pH (28). Our results show that the oxygen consumption measured with DP mme as a photosensitizer was considerably lower than when Sylsens B was used and it was influenced by the pH. In case of Sylsens B, the oxygen consumption was not affected by the pH.

	Percentage oxygen consumption /min	
pH	***Sylsens B***	***DP mme***
7.4	10 ± 2	2.3 ± 0.2
5.2	11 ± 3	0
3.5	9 ± 2	0

Table 5.1. Oxygen consumption, measured during the photo-oxidation of 4 mM tryptophan in the presence of 20 µM Sylsens B or DP mme and red light (30 mW/cm²) at pH 3.5, 5.2 and 7.4 (for pH 3.5 and 5.2, a 50 mM citric acid/sodium citrate buffer was used and for pH 7.4, a 50 mM sodium phosphate buffer was used); the values given are the means of three different experiments with the SD.

pH and positively charged molecules influence the binding of Sylsens B to T. rubrum

The results of the examination of binding capacities of the photosensitizers Sylsens B and DP mme to the fungal wall of *T. rubrum* at pH 5.2 and 7.4 are shown in Table 5.2. It can be seen that in the presence of a suspension culture of *T. rubrum*, 17 h of incubation with Sylsens B at pH 5.2 and 7.4 resulted in its binding to the fungus. A decreased fluorescence [significantly higher at pH 7.4 compared with 5.2; $t(6) = 3.119$, $P < 0.05$] was measured in the incubation medium after removal of the fungus, suggesting a part of the available Sylsens B was bound to the fungus. The same experiments performed after a 24 h pre-incubation of the fungus with the cationic dye AB (160 µM) or 5 mM $CaCl_2$, showed reduced binding of Sylsens B. The decrease was significant in the case of AB and pH 7.4 [$t(6) = 5.05$, $P < 0.05$]. Pre-incubation of *T. rubrum* with $CaCl_2$ resulted in a significant effect for both pH 5.2 [$t(6) = 3.184$, $P < 0.05$] and 7.4 [$t(6) = 6.274$, $P < 0.05$]. Moreover, incubating *T. rubrum* simultaneously with Sylsens B (160 µM) and $CaCl_2$ (5 mM) also resulted in a complete loss of binding of Sylsens B to the fungus [pH 5.2 $t(6) = 5.586$, pH 7.4 $t(6) = 6.90$; $P < 0.05$]. Furthermore, a binding to the SC was noticed for Sylsens B at pH 7.4 but not at pH 5.2.

Examining the binding of DP mme to the fungus under the given circumstances at both pH values, with or without AB pre-incubation, resulted in little detectable binding. In both cases, we saw a low decrease in fluorescence emission.

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	Fluorescence emission: percentage of control	
Incubation mixture (2 mL)	***pH 5.2***	***pH 7.4***
Water + 160 µM Sylsens B	100[a] ± 3 %	100[b] ± 6 %
Water + SC + 160 µM Sylsens B	99 ± 1 %	***79 ± 6 %***
Water + fungal culture + 160 µM Sylsens B	***73 ± 7 %***	***52 ± 11 %***
Water + fungal culture + 160 µM Sylsens B (pre-incubation with 160 µM AB)	87 ± 10 %	***86 ± 7 %***
Water + fungal culture + 160 µM Sylsens B (pre-incubation with 5 mM $CaCl_2$)	***87 ± 2 %***	***92 ± 1 %***
Water + fungal culture + 160 µM Sylsens B + 5 mM $CaCl_2$	***99 ± 4 %***	***96 ± 2 %***
Water + 200 µM DP mme	100[c] ± 2 %	100[d] ± 2 %
Water + SC + 200 µM DP mme	101 ± 6 %	***91 ± 6 %***
Water + fungal culture + 200 µM DP mme	***90 ± 2 %***	***90 ± 3 %***
Water + fungal culture + 200 µM DP mme (pre-incubation with 160 µM AB)	91 ± 2 %	90 ± 3 %

Table 5.2. Influence of AB and $CaCl_2$ on the fluorescence emission of Sylsens B at 657 nm (excitation 424 nm) and DP mme at 620 nm (excitation 392 nm) in the presence and absence of a *T. rubrum* suspension.
In all cases, 2 mL of a 5-day-old *T. rubrum* suspension culture was used and after washing away the medium, the fungus was taken up in 2 mL of water of the indicated pH. The influence of SC on the fluorescence emission of Sylsens B at 657 nm (excitation 424 nm) and DP mme at 620 nm (excitation 392 nm) is also included. The values given are the means of four different experiments with the SD. Values given in bold differ significantly (Student *t*-test, $P < 0.05$) from their control values.
[a] Control values for the fluorescence emission (AU) at pH 5.2: 99+3.
[b] Control values for the fluorescence emission (AU) at pH 7.4: 95+6.
[c] Control values for the fluorescence emission (AU) at pH 5.2: 329+7.
[d] Control values for the fluorescence emission (AU) at pH 7.4: 430+5.

Efficacy of the PDT of T. rubrum with Sylsens B depends on both the fungal growth stage and pH

Taking the MIC as a measure of the porphyrin efficacy, Fig. 5.4A shows that there is a difference in efficacy at various pH values when PDT is applied 17 h after spore inoculation. In this growth stage, PDT resulted in an MIC of 5 µM Sylsens B at pH 5.2, whereas at other pH values a shift to higher MICs was observed. It was also noticed that in the lower concentration range, the frequencies of the fungicidal effect at pH 5.2 and 3.5 were both significantly higher compared with pH 7.4. At 48 h after spore inoculation, we found no difference in MIC at all three pH values (Fig. 5.4B). However,

in the low concentration range there was a significant difference in the photodynamic effect when comparing pH 5.2 and 3.5 versus 7.4. The PDT results 72 h after spore inoculation (Fig. 5.4C) show that only in the case of pH 5.2, a significantly higher PDT effect was found for almost all test concentrations.

When comparing the PDT results obtained at different time points after inoculation, it can be said that the susceptibility of *T. rubrum* to PDT with Sylsens B declined at every selected pH when the time between the inoculation and the treatment increased.

The corresponding statistical data are given in Tables 5.3–5.5. As can be seen from Table 5.3, a main effect of the pH (10 µM Sylsens B) was found within every tested fungal growth stage. Within the 17 and 48 h fungal growth stage, significance was proven for both acidic pH values compared with pH 7.4 and within the 72 h growth stage, significance was proven for pH 7.4 and 3.5 compared with 5.2 (Table 5.4). In addition, within the 72 h growth stage, significance was proven for almost all other results obtained at pH 3.5 and 7.4 versus those obtained at pH 5.2, with the exception of 160 µM, pH 3.5 versus pH 5.2 (Wilcoxon signed-rank test, $P < 0.05$). Furthermore, within every pH a main effect of the growth stage was found (Table 5.5, Friedman's ANOVA, 10 µM Sylsens B).

Growth stage (h after spore inoculation)	***Test statistic χ^2 (ν [a])***	***P*** [b]
17	9.33 (2)	0.009
48	9.33 (2)	0.009
72	10 (2)	0.007

Table 5.3. Influence of pH (3.5, 5.2 and 7.4) on PDT efficacy within fungal growth stage according to Friedman's ANOVA (10 µM Sylsens B).
[a] ν, degrees of freedom.
[b] The critical level of significance used was 0.05 (*P* values given are two-tailed).

Test condition (growth stage,[a] pH)	***Test statistic Z***	***P*** [b]
17h, pH 7.4 versus 17h, pH 5.2	- 2.06	0.039
17h, pH 3.5 versus 17h, pH 7.4	- 2.12	0.034
48h, pH 7.4 versus 48h, pH 5.2	- 2.04	0.041
48h, pH 3.5 versus 48h, pH 7.4	- 2.12	0.034
72h, pH 7.4 versus 72h, pH 5.2	- 2.06	0.039
72h, pH 3.5 versus 72h, pH 5.2	- 2.06	0.039

Table 5.4. Results of the Wilcoxon signed-rank test to evaluate significance of the influence of pH and fungal growth stage on PDT efficacy.
[a] Hours after spore inoculation.
[b] The critical level of significance used was 0.05 (*P* values given are two-tailed).

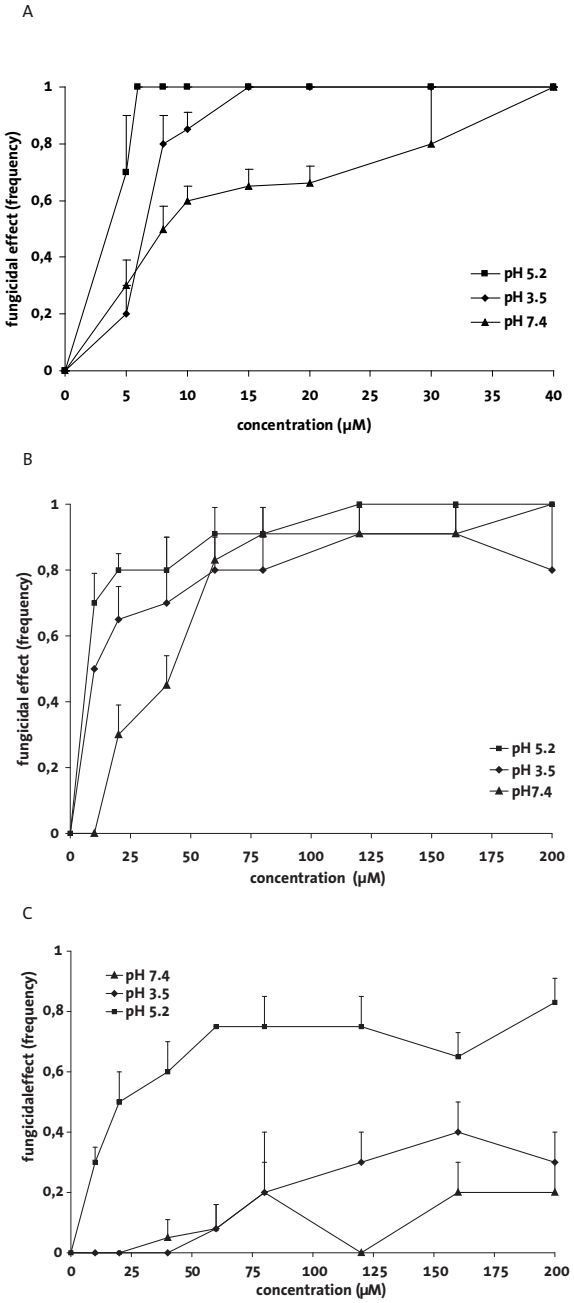

Figure 5.4. Photodynamic efficacy of Sylsens B towards *T. rubrum* tested at 17 (A), 48 (B) and 72 (C) h after spore inoculation on human SC, using a different incubation pH. Application of PDT was performed using the *ex vivo* model with a 2 h incubation period in distilled water of adjusted indicated pH, followed by 1 h of illumination (30 mW/cm^2, red light). Values given are the mean values for five different experiments and their SEM. For the 17 h stage, dark controls displayed 8–12 cfu. For the 48 and 72 h stage, dark controls displayed 8–12 cfu up to 80 µM Sylsens B and 5–8 cfu for higher concentrations. The light controls displayed 8–12 cfu for all stages.

pH	*Test statistic* χ^2 *(*ν [a]*)*	*P* [b]
3.5	10 (2)	0.007
5.2	7.68 (2)	0.021
7.4	10 (2)	0.007

Table 5.5. Influence of fungal growth stage (17, 48 and 72 h) on PDT efficacy within pH according to Friedman's ANOVA (10 μM Sylsens B).
[a] ν, degrees of freedom.
[b] The critical level of significance used was 0.05 (*P* values given are two-tailed).

Efficacy of the PDT of T. rubrum with DP mme depends on the fungal growth stage at pH 7.4 and is low at pH 5.2

Fig. 5.5 shows that the fungicidal effect of DP mme was clearly lower than that of Sylsens B. The reduction in pH from 7.4 to 5.2 resulted in every tested growth stage in a decrease of PDT efficacy. Similar to observations made with Sylsens B, the PDT efficacy decreased as growth stage increased and hardly any efficacy was found when PDT was applied 72 h after spore inoculation.

Higher buffer molarity is associated with lower PDT efficacy of Sylsens B

Fig. 5.6 shows a general tendency that a rise in buffer molarity decreases the PDT efficacy. This was observed especially in the 72 h growth stage. A large decrease in efficacy was visible already at the lowest buffer molarity (5 mM). A similar decrease was observed even when we used 0.5 mM concentration (data not shown). The further the fungal growth developed, the less susceptible the fungus was to PDT with Sylsens B under the conditions of increasing buffer molarity.

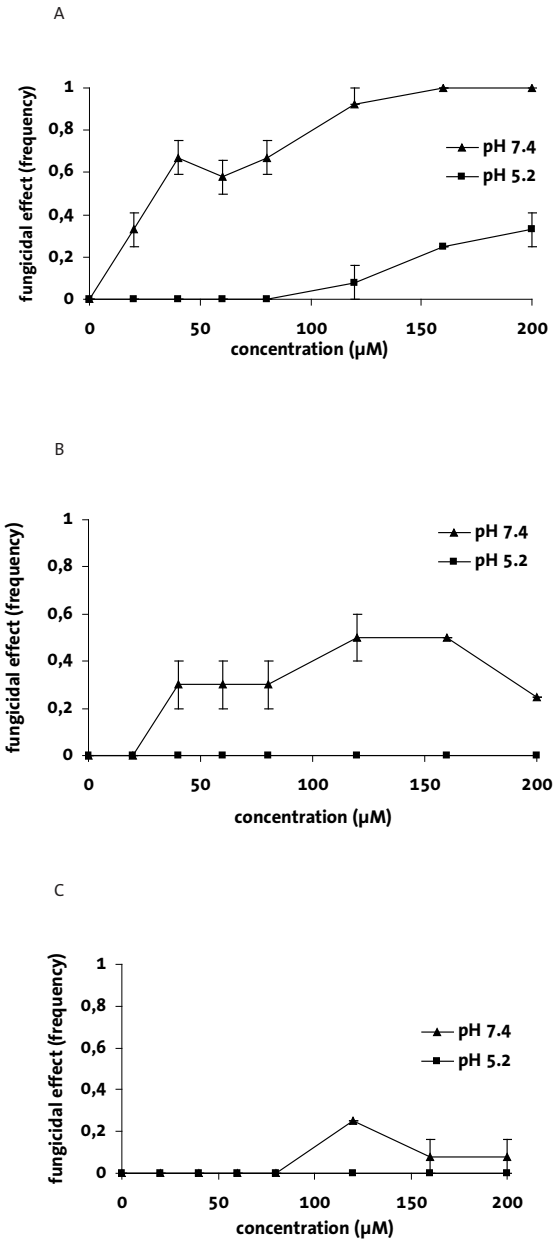

Figure 5.5. Photodynamic efficacy of DP mme towards *T. rubrum* tested at 17 (A), 48 (B) and 72 (C) h after spore inoculation on human SC, using a different incubation pH.
Application of PDT was performed using the *ex vivo* model with a 2 h incubation period in a 5 mM buffer solution of indicated pH (citric acid/ sodium citrate for pH 5.2 and sodium phosphate for pH 7.4), followed by 1 h of illumination (30 mW/cm2, red light). Values given are the mean values for three different experiments and their SEM. For all the growth stages, dark controls displayed 5–8 cfu, whereas light controls displayed 8–12 cfu.

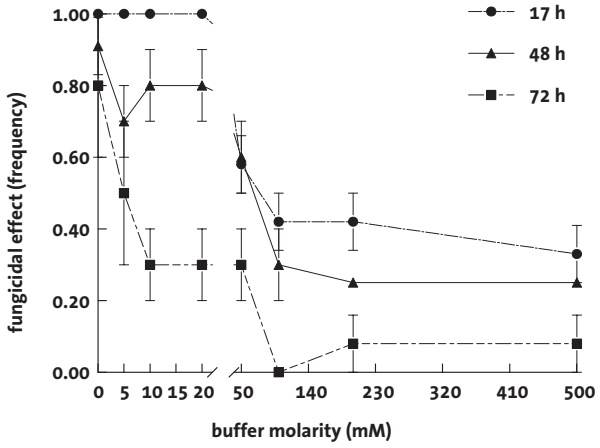

Figure 5.6. Photodynamic efficacy of Sylsens B towards *T. rubrum* tested at 17, 48 and 72 hours after spore inoculation using different molarities of a citric acid/sodium citrate buffer of pH 5.2.
Application of PDT was performed using the *ex vivo* model with a 2 h incubation period in a citric acid/sodium citrate buffer of pH 5.2 of different molarity, followed by 1 h of illumination (30 mW/cm^2, red light). The Sylsens B concentration was fixed at 10 µM for PDT application at 17 h after spore inoculation, 80 µM for application at 48 h after spore inoculation and 160 µM for application at 72 h after spore inoculation. Values given are the mean values for three different experiments and their SEM. Both light and dark controls displayed 8–12 cfu for all the growth stages.

Presence of PMSF increases the Sylsens B PDT efficacy at pH 7.4

The influence of some other PDT-effecting factors in the PDT efficiency is summarized in Table 5.6. In this table, the first four rows refer to the tests performed with 1 mM PMSF. This keratinase inhibitor was found to increase the Sylsens B efficacy significantly (Wilcoxon, z = -3.606, $P < 0.05$) for this 72 h stage at pH 7.4. When using only 40 µM Sylsens B in the absence of PMSF, the treatment resulted in an incomplete fungicidal effect (Fig. 5.4C and Table 5.6). The results were similar (Wilcoxon, z = -3.464, $P < 0.05$) when 60 µM Sylsens B was utilized (data not included).

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	Test substance concentration	*Photosensitzer concentration*	*Incubation pH*	*PDT application time*	*Fungicidal effect (frequency)[a]*
1	---	Sylsens B (40 µM)	7.4[b]	72 h after spore inoculation	1/20
2	5% propanol	0	7.4[b]	72 h after spore inoculation	0/10
3	PMSF (1mM) in 5% propanol	0	7.4[b]	72 h after spore inoculation	0/10
4	PMSF (1mM) in 5% propanol	Sylsens B (40 µM)	7.4[b]	72 h after spore inoculation	***14/20***
5	---	Sylsens B (10 µM)	5.2[c]	17 h after spore inoculation	12/12
6	---	Sylsens B (60 µM)	7.4[d]	17 h after spore inoculation	12/12
7	NaN_3 (2.5 mM)	0	5.2[c]	17 h after spore inoculation	0/6
8	NaN_3 (2.5 mM)	Sylsens B (10 µM)	5.2[c]	17 h after spore inoculation	***5/12***
9	Histidine (4mM)	0	7.4[d]/5.2[c]	17 h after spore inoculation	0/6
10	Histidine (4mM)	Sylsens B (60 µM)	7.4[d]	17 h after spore inoculation	***7/12***
11	Mannitol (10 mM)	0	7.4[d]/5.2[c]	17 h after spore inoculation	0/6
12	Mannitol (10 mM)	Sylsens B (10 µM)	5.2[c]	17 h after spore inoculation	12/12
13	Mannitol (10 mM)	Sylsens B (60 µM)	7.4[d]	17 h after spore inoculation	12/12
14	---	DP mme (160 µM)	5.2[c]	17 h after spore inoculation	2/12
15	---	DP mme (160 µM)	7.4[d]	17 h after spore inoculation	10/12
16	NaN_3 (2.5 mM)	DP mme (160 µM)	5.2[c]	17 h after spore inoculation	8/12
17	Histidine (4mM)	DP mme (160 µM)	7.4[d]	17 h after spore inoculation	***3/12***
18	Mannitol (10 mM)	DP mme (160 µM)	5.2[c]	17 h after spore inoculation	0/12
19	Mannitol (10 mM)	DP mme (160 µM)	7.4[d]	17 h after spore inoculation	12/12

Table 5.6. Influence of PMSF on the PDT efficacy of Sylsens B at pH 7.4 and the influence of sodium azide, histidine and mannitol on the PDT efficacy of both Sylsens B and DP mme (pH 5.2 and 7.4); values that differ significantly (Wilcoxon, $P < 0.05$) from the control values are given in bold.

[a] Number of fungicidal effects/number of tests.
[b] 5 mM sodium phosphate buffer.
[c] 20 mM sodium citrate acid buffer.
[d] 20 mM sodium phosphate buffer.

Sodium azide and histidine reduce the PDT effect of Sylsens B 17 h after spore inoculation

Seventeen hours after spore inoculation, a 100% fungicidal effect caused by 10 μM Sylsens B (at pH 5.2) was significantly reduced in the presence of 2.5 mM sodium azide (Wilcoxon, z = -2.530, $P < 0.05$). A comparable effect was also found in the presence of 4 mM histidine at pH 7.4 during illumination with 60 μM Sylsens B (Wilcoxon, z = -2.140, $P < 0.05$). We obtained similar results when we performed the experiments with DP mme. As expected, we found (Fig. 5.5C) only a small effect of this porphyrin (160 μM) at pH 5.2. At pH 7.4, the PDT efficacy of this photosensitizer was significantly (Wilcoxon, z = -2.646, $P < 0.05$) decreased when histidine was added during illumination.

DISCUSSION

We investigated several factors that may affect the PDT efficacy of Sylsens B and DP mme towards the dermatophyte *T. rubrum* when applied in different growth stages. The efficacy of Sylsens B was pH dependent for every tested growth stage with an optimum at pH 5.2. In case of DP mme hardly any effect was observed at acidic pH values and the maximum PDT effectiveness was observed at pH 7.4 at 17 h after spore inoculation. For both photosensitizers, a shorter growth stage resulted in a stronger PDT effect.

The central question in this study was: which conditions and mechanisms could be responsible for the differences in PDT efficacy of Sylsens B and DP mme? It is known that the pI of human skin is approximately pH 5 (29).Therefore, the SC will be negatively charged above this pH and the negative charge will be increased at pH 7.4. Our zeta potential measurements (Fig. 5.1) show that the fungal wall is negatively charged above pH 3.5. Comparing the pI from SC and fungal wall, it can be concluded that there is a narrow pH range (approximately pH 3.5–5.5) in which a selective binding of cationic Sylsens B molecules to the fungus can be achieved. In this pH range, the surface potential of the fungal elements is still more negatively charged than the SC and, consequently it can attract more positively charged molecules.

The best PDT effect for Sylsens B towards *T. rubrum* in the *ex vivo* model was obtained at pH of 3.5 or 5.2. This acidic pH effect was evident for every tested growth stage. However to cover all fungal growth stages, incubating at pH 5.2 should be preferred.

These observations fit very well with the idea of selective binding of Sylsens B to the

fungus. At this pH, the SC is almost neutral. In theory, however, the indicated selective binding can also take place at pH 3.5. Indeed, our experimental results obtained at pH 3.5 and 17 h after spore inoculation revealed an MIC of 15 μM, whereas at pH 7.4 it was almost three times higher. From the Zetasizer results, we found the pI for the fungal hyphae and microconidia to be approximately at pH 3.5.

This indicates that both the fungal hyphae and the microconidia are less negatively charged at pH 3.5, which corresponds to a lower binding of a positive-charged Sylsens B to the fungus and therefore a less efficient PDT.

The increased PDT efficacy can also be induced by the conversion of the photochemical properties of the photosensitizers as a function of the pH. However, in case of Sylsens B, we showed that the absorption and emission spectra as well as the $^1O_2$ production were not pH dependent. Therefore, the higher PDT efficacy of Sylsens B at pH 5.2 can be ascribed to the proposed selective binding.

The higher $^1O_2$ production measured for Sylsens B as compared with DP mme (Table 5.1) makes this photosensitizer a better candidate for PDT. This conclusion was reflected in outcomes from all PDT experiments. Furthermore, we observed a lower binding capacity of DP mme to the fungus and that both the absorption and fluorescence emission decreased remarkably at acid pH values. An increasing blue shift of the Soret band wavelength was observed for decreasing pH values, indicating that the aggregation of absorbing molecules proceeded as the pH decreased. Due to these observations, we only tested the PDT efficacy of this porphyrin at only one acidic pH 5.2. As expected, we found hardly any PDT effect. 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 uncharged DP mme molecules present, causing an increased affinity towards the hydrophobic SC. These factors will also account for a decreased PDT efficacy at pH 5.2.

We also observed a higher PDT efficacy when using both photosensitizers in the earlier growth stages. The lower PDT susceptibility was associated with progressing hyphae proliferation. Important in this aspect is also the short lifetime of singlet oxygen, which is 100 to 250 ns in a 'biological' environment (30-32) and the necessity of an effective photosensitizer binding for a successful PDT. Under the *ex vivo* conditions, the microconidia germination will probably be completed at 48 h after spore inoculation since at this stage we observed the appearance of fungal hyphae. The hyphae were not observed during the earlier growth stage at 17 h after spore inoculation. Although both fungal hyphae and microconidia have a negative surface potential of similar value, other morphological and structural

differences between the different tested growth stages could explain the obtained results. For instance, it is known that in many fungi, cell wall composition alters upon environmental changes (33).The wall structure of *T. rubrum* microconidia may differ from that of the hyphae (34). In previous studies, it was observed that the chemical composition of the fungal spore wall changed upon ageing (35). Another factor that is worth mentioning is the different wall structure at the fungal growth tops, the place of protein excretion. The binding ability at this terminal part of hyphae may differ from the rest of the fungal body. Unfortunately, no research has been performed on this subject in case of dermatophytes. But since we observed microscopically, that in case of an ineffective PDT re-growth of the fungus always occurred at one of the hyphae tips without recovering of the rest of the fungal body, we believe that this factor could be of importance. This subject is currently under investigation using scanning electron microscopy techniques.

Our binding studies involving Sylsens B and *T. rubrum* confirmed the higher binding capacity of Sylsens B at pH 7.4, in consistence with our zeta potential values. In a clinical situation, different kinds of cations may interfere with the binding of Sylsens B to the fungus. This is supported by the results of our binding assays (Table 5.2). Special attention was paid to Ca^{2+} ions because these ions are present at relatively high concentration in the upper epidermal layers. A future topical formulation will have to include a chelating agent.

The singlet oxygen scavengers, NaN_3 and histidine influenced the porphyrin PDT efficacy in a negative way, while mannitol did not have any influence. Therefore, hydroxyl radicals do not seem to be involved in the fungistatic effect, at least not in the fungal damage caused by the early PDT effect. However, we cannot completely rule out that the final fungal death could be caused by other reactive oxygen particles displaying a longer lifetime.

The use of an effective inhibitor of the *T. rubrum* keratinase activity increased the efficacy of the treatment. Inhibiting the keratinase activity could retard the spread of fungus and rendering this microorganism more susceptible to PDT.

All the results of our study will be of importance for the formulation of preparations that could be utilized for clinical PDT studies, rendering a valuable alternative for the treatment of tinea.

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