The susceptibility of trichophyton rubrum to photodynamic treatment

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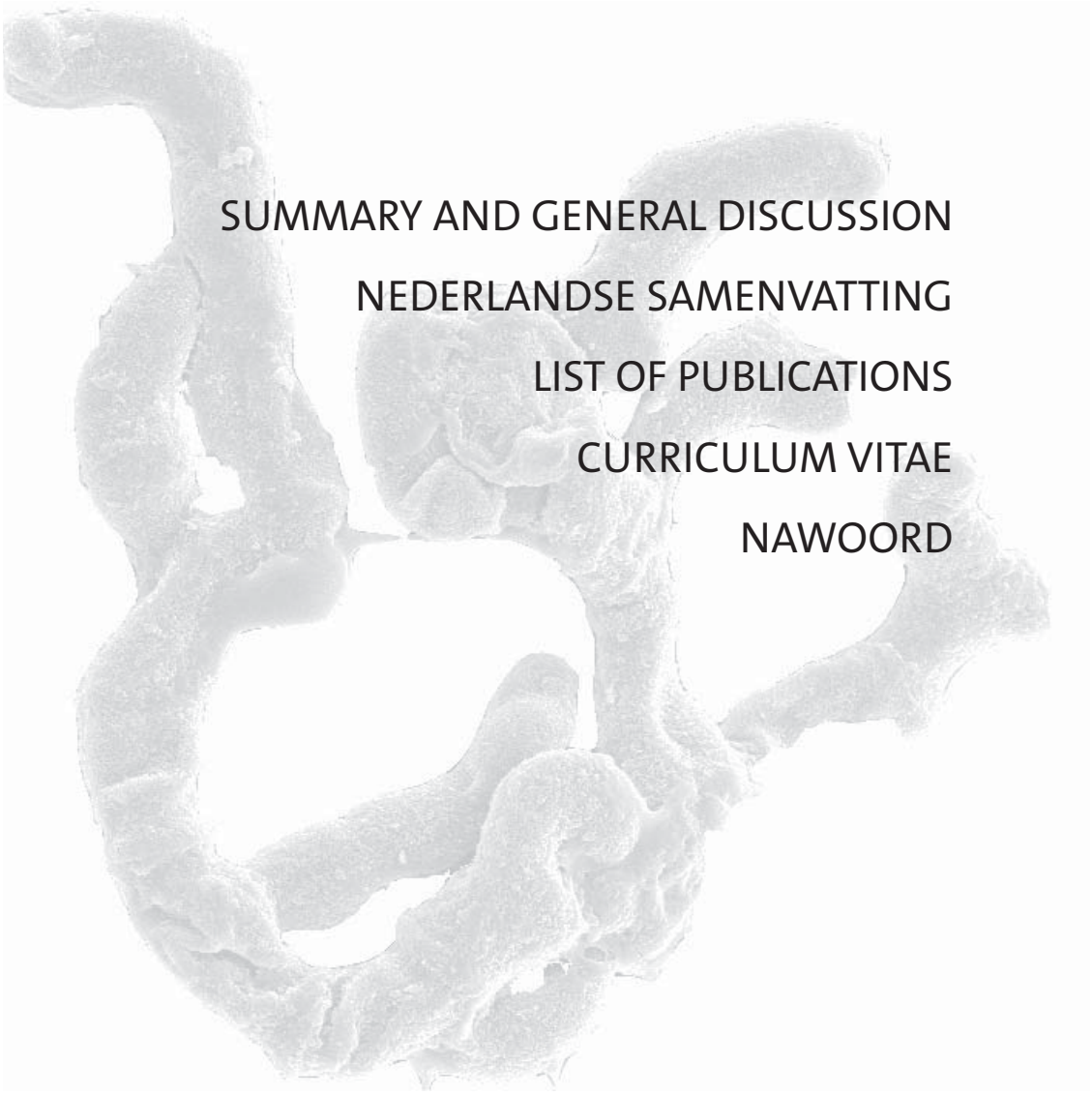
Chapter IX



SUMMARY AND GENERAL DISCUSSION

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Trichophyton rubrum mycelium destroyed by PDT with Sylsens B and red light at 48 hours after spore inoculation

SUMMARY AND GENERAL DISCUSSION

1.1 Summary

The main aim of this thesis was to investigate the susceptibility of the dermatophyte *Trichophyton rubrum* to photodynamic treatment (PDT), based on porphyrin photosensitizers, and to deduce a formulation suitable for one single effective treatment in a clinical application.

PDT refers to a treatment within a biological system with the use of light-activated agents, called photosensitizers, in combination with light of a proper wavelength and molecular oxygen (1,2). Commonly, in the presence of oxygen two competing photodynamic reaction types can occur (3,4). 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 singlet oxygen ($^1O_2$).

Since bacterial resistance to antibiotics has become a serious problem (5-7), search for new treatment possibilities is an important issue. It is generally agreed that $^1O_2$ is the key agent responsible for the cellular damage during antimicrobial PDT (8-10). As the lifetime of $^1O_2$ is very short, the binding of the photosensitizer to its target organism is crucial for an effective PDT (11,12).

Within the field of dermatology, the use of PDT has been studied intensively (13), predominantly on non melanoma skin cancer. This also includes the application for the superficial skin mycoses caused by dermatophytes (14-19), fungi that can cause infections of keratinized tissues. These infections are classified as tinea, according the location on the body. Several dermatophyte strains have been subjected to PDT using different kind of exogenous photosensitizers (thiophenes, methylene blue, phthalocyanines) and the endogenous protoporphyrin produced as a result of 5-aminolevulinic acid (ALA) PDT. However, a fungicidal effect has never been achieved in these studies.

The described investigations focus on *T. rubrum* because this dermatophyte is, worldwide, clinically the most frequently isolated strain (20-23). Moreover, the infections caused by this dematophyte (24,25) can be very persistent and therefore difficult to treat, partly because a decreased susceptibility to the host's immune response (26-29).

***T. rubrum* in suspension culture is susceptible to PDT with porphyrin photosensitizers (chapter 2)**

Because currently available therapeutic options for tinea have several limitations, this most frequently isolated dermatophyte was selected to study its susceptibility to PDT.

T. rubrum, cultivated in suspension culture, was subjected to PDT with the use of broadband white light. Various porphyrin photosensitizers were tested and their photodynamic activity compared to the activity obtained with several classical photosensitizers.

For the classical photosensitizers a fungistatic effect (a delay in growth) was observed, while the use of Deuteroporphyrin monomethyl ester (DP mme) and the cationic meso-substituted porphyrin 5,10,15-tris(4-methylpyridinium)-20-phenyl-[21*H*,23*H*]-porphine trichloride (Sylsens B) resulted in a fungicidal effect (a complete fungal kill).

Red light is effective in the porphyrin PDT of mycelium and microconidia suspensions of T. rubrum (chapter 3)

Dermatophytes often colonize both the stratum corneum (SC) and hair follicles. Therefore, photosensitizers absorbing in the red spectrum part are preferred as the red light penetrates sufficient deep, which makes it even possible for this light to penetrate the nail. This could offer a good perspective for the treatment of onychomycosis, the most prevalent infection of the nail.

The possibility of using red light for the PDT of *T. rubrum* (including the spores) with porphyrin photosensitizers was investigated *in vitro*. In addition to Sylsens B and DP mme, a newly synthesized quinoline porphyrin photosensitizer, quinolino-[4,5,6,7-efg]-7- demethyl-8-deethylmesoporphyrin dimethylester (QDD), was used. The quinoline porphyrin was included because this porphyrin had a distinct, high absorption peak in the red part of the spectrum.

However, the higher red light absorbing capacity of QDD did not result in a high photodynamic efficacy towards *T. rubrum* when using red light. Only a delay in growth was observed, while for Sylsens B and DP mme complete killing of both *T. rubrum* microconidia and hyphae was obtained.

Although it is known that QDD has a lower photostabilty, this will be of minor importance for the observed lack of PDT efficiency. More important could be its lower binding capacity for the fungal wall, due to the hydrophobic nature and charge characteristics of this molecule. Both Sylsens B and DP mme are, respectively positively

and negatively, charged. QDD is an uncharged molecule, lacking the capacity to bind sufficiently to *T. rubrum*.

A novel ex vivo model to investigate the photodynamic efficiency of porphyrins towards T. rubrum grown on human stratum corneum (chapter 4)

The successful PDT of *T. rubrum* with Sylsens B and DP mme in suspension cultures led to the question whether the same success could also be accomplished in experimental settings more closely resembling the clinical situation.

To address this issue, we developed an *ex vivo* model. In this model, the photodynamic activity of photosensitizers towards *T. rubrum* grown on human stratum corneum (SC) could be inverstigated. Moreover, the susceptibility of *T. rubrum* to PDT could be examined in different fungal growth stages, corresponding to different time points after the spore inoculation on the SC. Both Sylsens B and DP mme were used as photosensitizers in the model.

Compared to the *in vitro* studies, the susceptibility of *T. rubrum* to PDT with red light appeared to be lower than in the suspensions. Much higher photosensitizer concentrations had to be used to obtain complete fungal kill. Attachment of the fungus to human SC could play a role in the reduced efficiency of PDT. Moreover, a decrease in susceptibility was observed with increasing time of PDT application (after the spore inoculation) for both photosensitizers in Dulbecco's modified Eagle medium (DMEM). The fungal growth stage containing the spores attached to the SC (8 hours after spore inoculation) appeared to be more susceptible than the full mycelium stage (72 hours after spore inoculation).

Changing the incubation medium to distilled water increased the percentage of complete fungal kill by Sylsens B and decreased this percentage when DP mme was used. Factors like pH and ion strength of the water very probably account for the observed difference.

Fluorescence microscopic studies showed the presence of Sylsens B on the outside of the fungal wall in case of a dark (unsuccessful) photodynamic treatment with Sylsens B, but both inside and outside the fungal wall after a successful PDT.

The conclusion from this work was that PDT was successful in a situation that mimics the clinical situation. The fungicidal effect of PDT on fungal spores is of particular importance, as the fungal spores are generally resistant to treatment.

Low ion strength and pH increase the photodynamic efficacy of a cationic porphyrin (chapter 5)

In this study the key factors involved in PDT efficacy of both Sylsens B and DP mme were investigated in an *ex vivo* situation during different fungal growth stages. The study focused on the influence of pH and ion strength of incubation media, photochemical properties of the photosensitizers and phenylmethylsulphonylfluoride (keratinase inhibitor) on the PDT efficacy.

Based on the results thus obtained, a formulation was designed for optimal PDT of *T. rubrum* in experimental conditions that resembled the clinical situation.

The highest PDT efficacy for all tested fungal growth stages was obtained for Sylsens B at pH 5.2. Using these conditions for all tested fungal growth stages (corresponding from 8 to 72 hours after spore inoculation) complete fungal inactivation could be obtained. However, in case of treatment of the full mycelium (72 hours after spore inoculation) this fungicidal effect was obtained only in 80 to 90% of the cases.

An optimal pH of 5.2 and low concentrations of calcium are crucial for a selective binding of Sylsens B to the fungus, leading to an increased PDT efficacy. The proposed selective binding of Sylsens B to the fungus can be accomplished within a small pH range (pH 3.5 to 5.5). In this pH range the fungus will have a net negative charge, while the human SC will be more neutrally charged. This selective binding to *T. rubrum* cannot be accomplished for DP mme.

To conclude, 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.

Lethal PDT causes extensive fungal wall morphological alterations (chapter 6)

There have been only few reports describing morphological changes caused by PDT to microbes in general (30,31) and to *T. rubrum*, in particular.

We therefore visualized the effect of PDT to fungal wall morphology by scanning electron microscopy (SEM). Lethal and sub-lethal concentrations of Sylsens B were used. The morphological changes observed were compared to the changes caused by the light alone (108 J/cm^2 of red light) and the treatment of Sylsens B in the dark. The morphologic changes were examined at various time points after the treatment, applied to different fungal growth stages.

In general, in all the different fungal growth stages, the observed damage due to the different treatments occurred shortly after the treatment. The 108 J/cm^2 light dose in

the absence of Sylsens B and the application of this photosensitizer in the absence of light caused minor fungal wall deformations and bulge formation. Only after a lethal PDT, a sequence of severe disruptions and deformations of both microconidia and mycelium was observed. That resulted in the extrusion of cell material and emptied fungal elements. In case of a non-lethal PDT, fungal re-growth started on the remnants of the treated mycelium. Re-growth was observed mainly at hyphal tips. Under normal conditions we observed (at 72 hours after spore inoculation) a difference in morphology for the hyphal tips compared to the wall morphology of other hyphae parts. As a consequence, there may be a decreased binding capacity of Sylsens B to these hyphal tips resulting in a lower PDT efficacy.

Development of a photomuatgenicity test system (chapter 7)

Interest in potential application of photosensitizers for medical purposes increased remarkably during the last few years. Therefore, mutagenic potential of photosensitizers is an important issue to be investigated in a standardized, qualified test system for photomutagenicity. Up to now many different test systems for the detection of photochemical genotoxicity have been reported (32), but most of them have serious limitations. Based on the validated Somatic Mutation and Recombination Test, known as SMART and using *Drosophila melanogaster*, we developed the Photo-SMART and demonstrated that methylene blue (MB), known to induce photomutagenicity, can act as a positive control in the presented test system. The SMART scores for the loss of heterozygosity caused predominantly by homologous mitotic recombination. The photo-SMART can be utilized to detect photogenotoxicity of short-lived photoproducts and/or stable photoproducts. Using this method we found some indication for mutagenicity for Sylsens B. That was not the case when hematoporphyrin (HP) was examined.

The cationic porphyrin Sylsens B does not penetrate the skin barrier (chapter 8)

Currently available treatment modalities for tinea have certain limitations that are the cause of frequent disease recurrences (33,34). Moreover, it has been reported that the dermatophyte *T. rubrum* can reduce the host's immune response rendering it more resistant to the currently used treatments (27).

For a clinical treatment of tinea it is neither necessary nor desirable for Sylsens B to penetrate the skin.

In this final part of the research described in this thesis we investigated the skin

penetration behaviour of Sylsens B through dermatomed skin, human SC, disrupted human SC by *T. rubrum* growth and also through human SC pre-treated with a detergent. The effect of the optimal formulation for PDT (pH 5.2) on the Sylsens B penetration behaviour was investigated and compared to a formulation with PBS (pH 7.4). In addition, the penetration pattern of Sylsens B in healthy skin was visualised by confocal scanning laser microscopy. It was shown that, under the experimental conditions, no Sylsens B skin penetration occurred in healthy skin at pH 7.4 or 5.2. However, the preceding fungal growth caused Sylsens B penetration at pH 7.4 (PBS) but not in case of our successful PDT formulation with a pH of 5.2.

1.2 General discussion and conclusion

The research work described in this thesis resulted in a formulation that may be used for the single photodynamic treatment of tinea caused by *T. rubrum*. The pharmaceutical composition of Sylsens B solution should have a pH preferable between 4.5 and 5.7, low ion strength (max. 5mM), and contain a chelating agent (35).

With this formulation, PDT can effectively kill *T. rubrum ex vivo* with a red light dose of 108 J/cm^2. Of remarkable importance is the effectiveness of the treatment towards the spores of *T. rubrum*. Since currently available treatments preferentially affect the metabolic active fungal forms, the spores usually remain undisturbed in the skin. This can be the beginning of renewed infection. This is the most important cause of the high recurrence percentage of tinea. The higher PDT efficacy of Sylsens B to, in particular, *T. rubrum* microconidia could be ascribed to their smaller size. Since effective binding of the photosensitizer is a requirement for a successful PDT, the photosensitizer can more easily occupy the smaller surface area of the microconidia than the larger area of a full mycelium. Additionally, the difference in wall pigmentation between conidial and hyphal fungal elements might play a role (36) as the presence of other light absorbing components could contribute to the overall fungicidal PDT effect. At last, microconidial wall morphology appears to be uniform. That could improve the change of Sylsens B binding. The thickness of the microconidia wall will be of minor importance, because prominent in an effective PDT of *T. rubrum* is the binding of Sylsens B to the outer fungal wall.

It appeared that the full mycelium present at 72 hours after spore inoculation was more difficult to treat. PDT with 160 µM Sylsens B in combination with red light resulted in only 80 to 90 percent of the cases in a fungicidal effect. In the other 10 to 20 percent of the cases fungal re-growth was observed.

The high effectiveness of the cationic photosensitizer Sylsens B when compared to negatively and neutrally charged photosensitizers is in accordance with previous antimicrobial PDT studies. We observed hardly any efficacy for the neutral porphyrin quinolino-[4,5,6,7-efg]-7-demethyl-8 deethylmesoporphyrin dimethylester (QDD), despite its excellent absorbing capacity in the red light spectrum part. Moreover, within the charged porphyrin photosensitizers, the cationic Sylsens B appeared to be a better photosensitizer towards *T. rubrum*, than the anionic DP mme.

In general, Sylsens B binding to the outer fungal wall in the dark may cause a wall destabilization, rendering it more susceptible to the $^1O_2$ produced by a subsequent PDT. Upon illumination, preceding fungal wall damages causes Sylsens B to enter into deeper wall layers and the interior of the fungus. Eventually, continuing illumination and $^1O_2$ production causes fungal death.

The binding of Sylsens B to *T. rubrum* wall must be based on ionic interactions as the competition with Ca^{2+} was observed. Identification of the binding sites for Sylsens B was not investigated within this thesis, though based on our mechanistic study we speculate that phosphate units of phosphoproteins and mannosylphosphate residues, could be responsible for the binding to the fungal wall. In other fungi (*Saccharomyces cerevisiae*) presence of mannosylphosphate groups in the outer glucan wall layer was proven (37,38). These residues provide the cell wall with a net negative charge. Moreover, the affinity of positively charged Alcian Blue (AB) to the fungal wall, as observed for *T. rubrum*, was also reported for other fungi. In these studies the mannosylphosphate units present in the outer wall of Saccharomyces cerevisiae were identified as binding sites for AB (37,38). Furthermore, our own experiments proved that binding of AB to *T. rubrum* decreased the binding of Sylsens B to the fungus.

Finally, the selective binding of Sylsens B to the fungus in the optimized formulation may prevent penetration of the photosensitizer through the skin, and it may also prevent healthy superficial skin layers from undesirable PDT effects.

The new treatment strategy for tinea caused by the dermatophyte *Trichophyton rubrum* that is based on photodynamic principles is covered by two patents (35,39). This will be important for further commercial exploitation.

1.3 Perspectives

According to the results obtained in the described studies, there are still several options that can be used to increase the efficacy to a 100 % fungicidal effect. Addition of a keratinase inhibitor to the incubation mixture is one possibility, as it is known to increase the Sylsens B PDT efficacy. Some earlier studies demonstrated the importance of proteolytic enzyme activity for fungal development and growth (40-42) in the presence of keratin as a sole nutrient. According to the results obtained during our SEM investigations, optional PDT repetition within 48 hours after the first application could also be used to increase the efficacy. This assumption has been studied recently and it showed that repetition of the PDT within 17 hours with 160 μM Sylsens B resulted in 100% fungicidal effect (unpublished results). A third, and more desirable option for the planned clinical PDT studies would be optimization of the light source. Up to now a broad range within the red light spectrum was used. From the absorption spectrum of Sylsens B shown in Fig. 9.1 it is clear that only the small Q2 (589 nm) and Q1 (649 nm) bands account for the obtained fungicidal effect in our studies. This indicates that optimization of the light source that would include the Soret band absorption could lead to improved PDT efficiency. The preliminary results, using UVA-1 light (340 - 550 nm) are very promising. Consequently, this may also reduce the required Sylsens B concentration. Another possibility worthwhile exploring is the use of a green light source covering the wavelength range of the Q4 band (514 nm). Additional advantage of this option would be that the penetration capacity of this light would be high enough to penetrate thick keratotic layers like the nail. This is of particular importance for the development PDT for onychomycosis. This is the most prevalent infection of the nail, mainly caused by the dermatophyte *T. rubrum*.

For our future research, we have planned the development and synthesis of new cationic porphyrin photosensitizers. We will use these porphyrins, together with Sylsens B, to investigate the possibility of PDT for fungal infections of skin, hair and nails caused by *T. rubrum* and other dermatophytes.

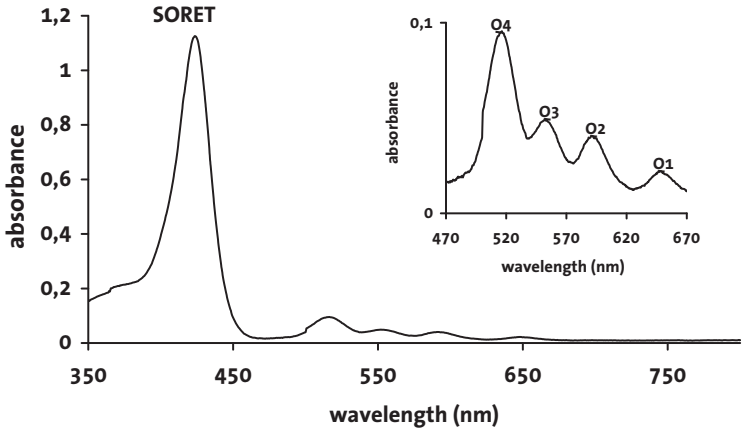

Figure 9.1. Absorption spectrum of Sylsens B in methanol.

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