The susceptibility of trichophyton rubrum to photodynamic treatment

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Une idée que j'ai, il faut que je la nie:
c'est ma manière de l'essayer
(Histoire de mes pensées, Émile Augiste Chartier, 1868-1951)

A mon père inconnu, peu m'importe la personne qu'il soit et où il se trouve

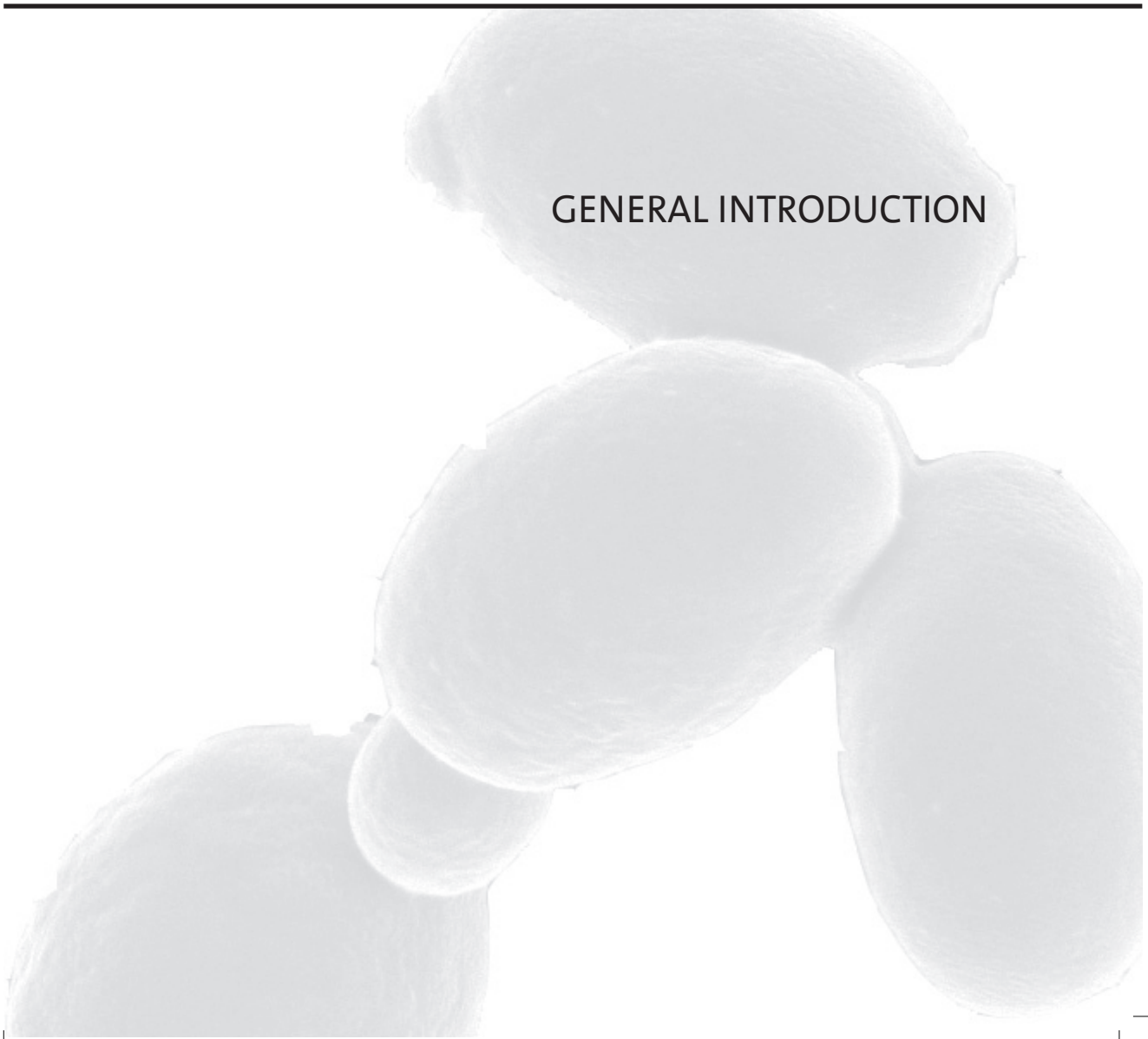
ABBREVIATIONS

ALA	5-aminolevulinic acid
AB	Alcian blue
$AlPcS_4$	Aluminium (III) phthalocyanine chloride tetrasulfonate
CBS	Centraalbureau voor Schimmelcultures
CFU	Colony forming units
DIC	Differential interference contrast
DP	Deuteroporphyrin
DP mme	Deuteroporphyrin monomethylester
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethylformamide
FCS	Fetal Calf Serum
Hp	Hematoporphyrin
HpD	Hematoporphyrin derivative
MEA	Malt Extract Agar
LOH	Loss of heterozygosity
MB	Methylene blue
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resitant *S. aureus*
$^1O_2$	Singlet oxygen
O_2^-	Superoxide anion radical
PcS_4	Phthalocyanine tetrasulfonate
PDT	Photodynamic treatment
PMSF	Phenylmethylsulphonylfluoride
Pp	Protoporphyrin
QDD	Quinolino-[4,5,6,7-efg]-7-demethyl-8-deethylmesoporphyrin dimethylester
ROS	Reactive oxygen species
SC	Stratum corneum
SD	Standard deviation
SEM	Standard error of mean
SMART	Somatic Mutation and Recombination Test
Sb	*Stubble*
TB	Toluidine blue
Tris	Tris(hydroxymethyl)aminomethane
ZnPc	Zinc phthalocyanine

CONTENTS

Chapter I

GENERAL INTRODUCTION



Trichophyton rubrum microconidia, 8 hours after inoculation on human stratum

1 PHOTODYNAMIC TREATMENT

1.1 Historical perspective within dermatology

The concept of photodynamic treatment (PDT) refers to a treatment with the use of light-activated agents, referred to as photosensitizers, in combination with light of a proper wavelength and molecular oxygen (1,2). Although the original use of photosensitized chemicals (obtained from plant extracts) for the treatment of skin diseases was already known from the ancient Egypt and Greece (3), the initial use of this treatment is usually ascribed to the work of Oscar Raab, as he was the first who published on the subject in detail (4). He reported the rapid killing of the protozoan, paramecium, upon illumination of dyes like acridine orange. Oscar Raab was a medical student who worked under supervision of Professor Hermann von Tappeiner. Von Tappeiner himself performed thorough investigations, discovered the presence of oxygen as a precondition required for the light reaction to occur and introduced the term photodynamic action (5,6). In a short time, the application of PDT for tissue destruction commenced and, in the same period, Jesionek and von Tappeiner reported the successful use of 5% eosine in the treatment of skin cancer (7). Soon after these pioneer studies, in 1909, the photosensitizing properties of the porphyrin hematoporphyrin were discovered (8). In these early days, the administration of hematoporphyrin was only systemic (9) to ensure a good uptake in different tumours and, consequently, an effective PDT. A large disadvantage of this systemic porphyrin PDT was the lasting cutaneous photosensitivity (10) which, however, stimulated interest in topical application of photosensitizers. The increasing use of clinical application of PDT was mainly inspired by the work of Dougherty and colleagues (11,12). From that period, the popularity of PDT as a treatment option for malignant tumours has grown enormously (13-15). This is reflected by a growth of the number of publications from this area (16-20).

Mainly due to the accessibility of skin to light, scientists have intensely investigated the use of PDT for both skin cancers and other, non-malignant skin conditions (3). As a result, not only oncologic but also non-oncologic applications of PDT are nowadays recognized in dermatology (12,14,19,21).

The non-malignant skin disorders that have been (experimentally) treated with PDT include psoriasis, lichen ruben planus, lichen sclerosus et atrophicus, scleroderma, alopecia areata, human papillomavirus infections and Darier's disease (3,14,22-24). Another important development in dermatology concerns the usage of PDT in bacterial and fungal skin infections (25-30).

1.2 Mechanism

1.2.1 Light

Light can be defined as a natural phenomenon that can be described by means of an electromagnetic wavelike concept or particle concept. In the particle concept, light is represented by a flow of particles with energy levels at very discrete values, initially described by Planck as light quanta (31) and later by Lewis as photons (32). According to Planck's law, the energy corresponding to one photon is given by:

$E = h\nu = hc/\lambda$
c = velocity of light (3×10^8 m s^{-1})
h = Planck's constant (6.63×10^{-34} J s)
λ = wavelength (m)
ν = light frequency (s^{-1}).

The spin multiplicity, an important aspect of molecules that can attain various energy states, is given by the relation M = 2S + 1. The total spin of state, S, can be found by adding the individual electron spin quantum numbers, S_1 and S_2. In most molecules in the ground state, the highest occupied molecular orbital contains 2 electrons with opposite spin (spin quantum number of +1/2 and −1/2) and the total spin equals 0. In this case the spin multiplicity is 1 (a singlet, ^1S, state). If a molecule (^1S) absorbs light, one of the electrons may be promoted to a higher excited state. According to Wigner's rule, the spin is conserved and the excited state is a singlet-excited state ($^1S^*$).
Subsequent to the excitation, one of the following events may occur:

1) Emission of a photon
2) Conversion to a different state. If there is no change in spin multiplicity, the change is called *internal conversion*. In case of an alteration of the spin multiplicity the term *intersystem crossing* is used
3) A chemical reaction (see Fig. 1.1 for the possible photochemical reaction types)
4) An electronic excitation.

The chemical reaction (Fig. 1.1) that is important in PDT is photosensitization.

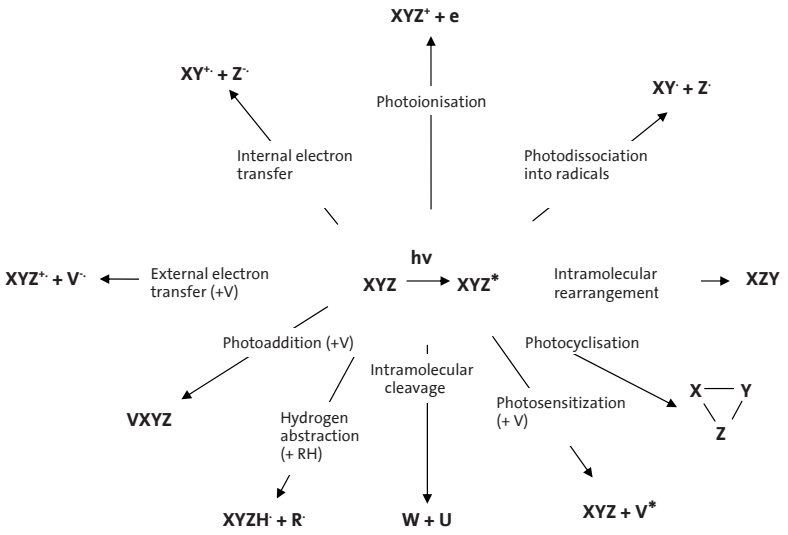


Figure 1.1. Important photochemical reaction types following excitation. The excited state, XYZ* , may be either singlet or triplet.

1.2.2 Photodynamic action

The term "photodynamic" refers to those photosensitized reactions that require molecular oxygen and occur within biological systems (6).

Following the absorption of light the photosensitizer is transformed from its ground state (S) to an excited singlet state (^{1}S). By intersystem crossing the short-lived singlet excited state is transformed to the triplet excited state (^{3}S). Subsequently, the excited triplet state can undergo two kinds of chemical reactions. The reaction types are commonly referred to as a *type I* and a *type II* reaction (see Fig. 1.2) (33,34). In a *type I* reaction, the triplet state activator ^{3}S* can either abstract an electron from, or donate an electron to a substrate molecule (A). The sensitizer radicals ($S^{-\cdot}$ and $S^{+\cdot}$) can react in a way to regenerate the ground state sensitizer, while substrate radicals can react with other molecules to give oxidized forms of the substrate. Interaction of the anionic state of some sensitizers with oxygen can give the superoxide radical ($O_2^{-\cdot}$) which can react with various types of biomolecules (35). In a *type II* reaction the triplet excited state sensitizer (^{3}S*) reacts directly with ground-state oxygen ($^3O_2$) (1). Provided the energy difference between ^{3}S* and the ground state of the photosensitizer (^{1}S) exceeds 94.5 kJ (the energy difference between $^3O_2$ and $^1O_2$), an allowed energy transfer leads to the formation of an excited singlet state of oxygen ($^1O_2$).

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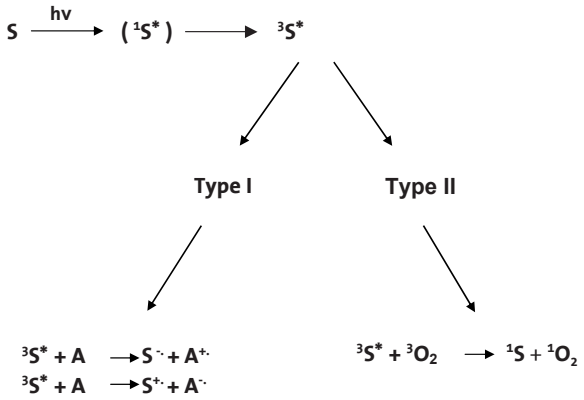

Figure 1.2. Illustration of the photodynamic reaction types that may occur during photodynamic action.

Singlet oxygen is a powerful, short-lived, electrophilic particle and reacts rapidly with electron-rich molecules that can be present in a variety of biological molecules and assemblies (12,36). The lifetime of $^1O_2$ in water is 3-4 µs whereas in organic solvents the lifetime is about 4-50 times longer (34). However in cellular systems the lifetime of $^1O_2$ is considerably shorter, namely 100-250 ns (37). Thus the site of the generation of $^1O_2$ determines which cellular structures may be attacked. Although most photosensitizers can react both via charge transfer and energy transfer reactions, it is generally agreed that singlet oxygen is the key agent of cellular damage (34). Although the *type II* mechanism predominates over the *type I* mechanism it cannot be excluded that $O_2^{-\cdot}$ may also be involved in PDT damage (34,38-40).
Ideal photosensitizers for PDT have the following properties (34,41-46).

- Chemical purity, photo-stability and constant composition.
- A high singlet oxygen yield.
- The ability to absorb light at the long wavelength range. Since both absorption and scattering of light by tissue decreases as wavelength increases, the most efficient photosensitizers have strong absorption bands in the wavelength range above 600 nm.
- Minimal dark toxicity.
- Low photo-toxicity.
- Good solubility in injectable solvents
- A low-cost large-scale production.

1.2.3 Porphyrin photosensitizers

Compounds that have been extensively used as photosensitizers are porphyrins. These compounds all contain a porphine macrocyclic ring, (Fig. 1.3). Porphyrins have very specific features that make them particularly useful as photosensitizers in biological systems:

- Porphyrins absorb light in a broad-spectrum range.
- Porphyrins normally have long-lived triplet states with high quantum yields (>0.7). This triplet state, the number of the singlet excite state sensitizer molecules that cross over to the triplet excites state, of porphyrins is successfully quenched by oxygen. This makes porphyrins typically *type II* photosensitizers causing cell damage through the generation of singlet oxygen (47).
- Porphyrin compounds can be synthesized and modified on demand. This offers the possibility to adjust the physico-chemical properties of the porphyrin molecules and control the positioning among subcellular compartments.

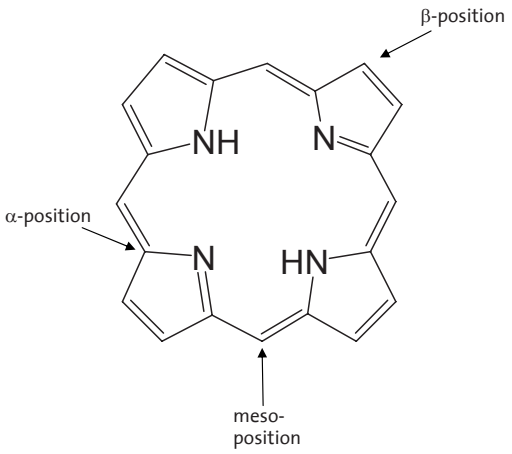

Figure 1.3. Structure of the unsubstituted porphine macrocycle ring.

Among the porphyrin photosensitizers used in medical PDT, hematoporphyrin (Hp) and its complex mixture of porphyrin derivatives (HpD) have been studied most intensively (11,36,48-51). In 1913 Meyer-Betz was the first to show photosensitization by Hp in man (52). Ten years later Policard observed the tendency of porphyrins to accumulate

in tumor cells (53) and in 1942 Auler and Banzer showed that Hp accumulation in tumors caused photonecrosis (54). Hp was first synthesized in 1960 (48) and from 1960 to 1983 Hp and HpD were intensively used as photosensitizers in PDT. These porphyrins are usually designated as the first generation of photosensitizers (46,55). Although, Dougherty et al. successfully treated tumours in mice and rats using HpD PDT already in 1975 (56), it was not until 1987 when the commercial available form of HpD, named Photofrin, became available (57,58). Despite the success of HpD, there were serious limitations in the use of these photosensitizers. HpD is a complex mixture, consisting of both photoactive and non-photoactive compounds (48,55,59). Moreover, its composition is difficult to reproduce (46,60-62). Therefore, to improve the efficacy of PDT, a second-generation photosensitizers, including new porphyrins, has been developed (21,45,55,63,64). The most important characteristics of these compounds that make them excellent photosensitizers are the strong absorption band in the red part of the spectrum, the ability to generate a high $^1O_2$ yield and the lack of dark toxicity (45,55,63,65,66).

Another interesting development is the use of the endogenous photosensitizer, protoporphyrin (Pp), produced from its precursor 5-aminolevulinic acid (ALA) in the heme biosynthesis (12,42,45,46,67-72). Since the conversion of Pp into heme is slow, Pp can accumulate in cells upon their exposure to ALA. A commercial form for ALA, Levulan Kerastick was approved by the Food and Drugs Administration (FDA) in September 2000 (73) and in 2004 methyl 5-aminolevulinic acid (Metvix) was approved for the pre-cancer actinic keratoses (74).

The most novel category of photosensitizers, including porphyrins, that may be used for PDT in future comprises completely synthetic second-generation photosensitizers (45,63,75-81). Among the porphyrins, the meso-substituted porphyrins have been developed as particular interesting photosensitizers (45,63,77,82-86). In general, the cationic photosensitizers, including the meso- substituted porphyrins, appear to be more photoactive than the anionic ones (82,84,87-91).

1.3 Antimicrobial PDT

1.3.1 General introduction

Today, bacterial resistance to antibiotics is worldwide an increasing concern (92-94). Many human pathogens are now resistant to many antimicrobial drugs. This makes the treatment of microbial skin infections sometimes difficult. The gram-positive *Staphylococcus aureus* and *Streptococcus pyogenes* and the gram-negative

Pseudomonas aeruginosa are important causes of various bacterial skin infections (94-99). It was already in the late 1950's that 50% of all S. *aureus* strains were resistant to penicillin (98). Moreover, the methicillin-resitant *S. aureus* (MRSA) is since 1980 a problem (100). PDT could offer new and safe perspectives for the treatment of localized bacterial skin infections, including wound infections (91,101).

In case of anti-microbial PDT the photodynamic effect depends mainly on the physical and chemical properties of the photosensitizer, such as the maximum absorption wavelength, the molar extinction coefficient and the $^1O_2$ production (82,85,91,102-104). The chemical properties determine the binding affinity of photosensitizer to the surface of the microorganisms. Because the surface of bacteria is negatively charged, positively charged photosensitizers are commonly more effective than those that have a negative or no charge. Generally, gram-positive pathogens are more susceptible to PDT than gram-negative (88,89,91). So, the chemical structure and the morphology of the cell wall is an important determinant here in PDT efficacy (90,91,105). In general, when the photosensitizer does not penetrate the outer wall membrane antimicrobial PDT is thought to occur via a *type II* mechanism and when it is penetrating the cell PDT is likely to occur via a *type I* (91).

1.3.2 PDT of superficial fungal skin infections

Superficial skin mycoses either caused by the yeast *Candida* or by dermatophytes (dermatophytoses) are the most common of human infections (27,106-111). Dermatophytes are fungi that can cause infections of keratinized tissues of the skin, hair and nails because of their ability to feed on keratin (112-115). The most important limitations of the current therapeutic treatments for superficial mycoses are the frequent recurrences of the infection and the duration of the treatment (116-119). This demands strongly for new therapeutic options and PDT belongs to the new promising treatment possibilities (27). However, the reports about the successful application of PDT on fungal skin infection are still very scarce.

In case the fungal skin infection does not invade the stratum corneum (SC), the light used for antifungal PDT may be in the blue spectrum region. However, as dermatophytes often colonize both the SC and the hair follicles, photosensitizers absorbing in the red and near infrared spectral part are preferred, because light of longer wavelength exhibits better penetration than blue light. The photosensitizers studied for superficial fungal skin infections mainly comprise phenothiazine dyes, phthalocyanines and porphyrins (27).

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It has been reported that *Candida* could be effectively killed by PDT with one of the phenothiazine dyes, methylene blue (MB) or toluidine blue (TB) (101,120). The efficacy appeared to be less compared to PDT of several prokaryotic bacteria but high when compared to the killing of keratinocytes (101,121). Recently, it was also demonstrated that *Candida* was susceptible to PDT with the porphyrin 5-phenyl-10,15,20-tris(N-methyl-4-pyridyl)porphyrin chloride. Candida could be successfully inactivated *in vitro* by this cationic porphyrin photosensitizer (84). As regards the dermatophytes, several strains (*Trichophyton mentagrophytes*, *Trichophyton rubrum, Trichophyton tonsurans, Microsporum cookie, Microsporum canis, Microsporum gypseum, Epidermophyton floccosum, Nannizia cajetani)* were exposed to UVA during the incubation with two different thiophenes (122). Although a strong dose-dependent growth inhibition could be observed, a fungicidal effect was not achieved in this study. Furthermore, Propst and Lubin reported the *in vitro* and *in vivo* (using albino guinea pigs) photosensitized killing of *Trichophyton mentagrophytes* and *Microsporum gypseum*. The authors used as a photosensitizer MB and proflavine dye, but the effective fungal kill observed in the *in vitro* tests could not be reproduced in the *in vivo* studies (25). Finally, the use of ALA-PDT is worth mentioning. Kamp and co-workers used ALA-PDT for the treatment of *Trichophyton rubrum* in liquid culture and reported a fungal growth inhibition of 50% (28). In addition, Calzavara-Pinton *et al*. observed a good therapeutic effect by ALA-PDT on interdigital mycoses of feet, but unfortunately the treatment could not prevent quick recurrences (26).

2 THE DERMATOPHYTE *TRICHOPHYTON RUBRUM*

2.1 Introduction to dermatophytes

Dermatophytes are traditionally divided in three anamorphic genera, *Trichophyton*, *Microsporum* and *Epidermophyton*. Although some *Trichophyton and Microsporum* species may have a sexual (teleomorph) stage named *Arthroderma*, their role in epidemiology of mycoses has not been established (123). Therefore, in medical mycology the 3 genera are classified as the anamorphic class Hyphomycetes of the Deuteromycota (Fungi Imperfecti) (113,124). Two important features contribute to the manifestation of a disease caused by the dermatophytes, namely the ability to feed on keratin-rich substrates and host specialization (123,125). In interactions between host and dermatophyte,

signals from the host can alter the dermatophyte's gene expression (126). In a clinical situation, persistent spores (arthroconidia or chlamydospores) are firmly attached to dermatophytes. These structures, embedded in hair or skin scales are heat resistant and may persist for many years (113,127-130). When germination takes place epidermal invasion will follow and dermatophyte hyphae will grow and penetrate the epidermal or hair structures (131-134). Keratinase enzyme production, induced by the presence of keratin, is thought to play an important role in the hyphae penetration during fungal invasion (125,134). Infections caused by dermatophytes are classified as dermatomycoses, or more correctly dermatophytoses (also named tinea), and their detailed name reflects the location on the body, e.g. tinea capitis (scalp) and tinea pedis (feet) (109,135,136). The treatment involves the use of an antifungal drug in either a topical or oral application (116,119,137) or a combination of both (117). The frequently used drugs can be divided into three different classes, the polyenes, the imidazoles and the allylamines (111,138). Apart from these classes griseofulvin (111) and cyclopiroxolamine (139) are occasionally used. Many of these antimycotics inhibit the synthesis of ergosterol, one of the building blocks of fungal membranes. This inhibition is only possible in growing microorganism. That is why the effect of many current antimycotics on spores is insufficient, leading to relatively frequent recurrences and the necessity of lengthy treatment (118).

2.2 Trichophyton rubrum

Trichophyton rubrum (Castellani) Sabouraud was first isolated from humans in 1910 by Bang (140-142). Currently,*T. rubrum* is the anthropophilic fungus that is most frequently isolated from patients suffering from mycotic skin diseases like tinea pedis, tinea corporis (107,143-146) and from tinea unquium (onychomycosis), representing the most prevalent nail infection (147).The infections caused by this world-wide distributed dermatophyte (135,148) can be very persistent and therefore difficult to treat, partly because a decreased efficiency of the host's immune response (149-152). As mentioned before, the present treatment strategies mostly affect the metabolic active fungus, not effecting the spores (116-118,138).

T. rubrum has several distinct colonial forms characterized by differences in produced pigments (153). When cultivated *in vitro*, both single cell microconidia (peg-shaped) and 1-8 celled macroconidia (cigar shaped) are produced. In *in vivo* situation *T. rubrum* mainly produces arthroconidia (brick-shaped) and to a lesser extent microconidia (154-156).

There are different reasons why *T. rubrum* can cause chronic infections in humans (150,157,158). First, in many patients the cell-mediated immunity to the dermatophyte antigen part that is specific to *T. rubrum* is lacking, due to differences in antigen skin penetration. Second, cell-wall components, like galactomannans may have immunosuppressive effects, inhibiting normal immune reactions (like lymphoprolifiration). Third, the ability of *T. rubrum* to evade host defense systems by remaining in superficial skin layers is also considered to be of importance. Finally, the possibility of *T. rubrum* to survive both in and off skin as a spore accounts for the high prevalence of infections caused by this fungus (150,159).

In the infection process the fungal wall (see Fig. 1.4) plays an essential role and is therefore also the target of many antifungals (111,117,160-162). The outermost layer of the wall constitutes of β-glucan, composed of α-glucopyranose units with predominantly β-1,3 - and β-1,6 -linkages (123,162-164). The second layer contains galactomannans, complex glycoproteins consisting of α - mannopyranose, mannofuranose, galactofuranose attached to a peptide backbone (150,158,162). In general *Trichophyton* species have two kinds of galactomannans, i.e. galactomannan I and II. In *T. rubrum* in galactomannan I the galactofuranose units are missing and galactomannan II contains α-1,2 en α-1,6 - linked mannopyranose and mannofuranose units (162). The third layer is known as chitin, a β-1,4 linked polymer of *N* -acetylglucosamine, giving the fungal wall its rigidity (123). The innermost layer is the cell membrane containing lipids, proteins and little carbohydrates. It resembles the cell membrane in eukaryotic cells except that in case of *Trichophyton* cholesterol is replaced by ergosterol. Due to this layered structure, the total wall thickness is approximately 100 to 300 nm, but it is thinner at the growing hyphal tips (123,165). In addition, dermatophyte hyphal walls contain relatively high levels of (negatively charged) phosphoproteins, potassium and sodium (160).

Although there are no reports on the wall architecture of the *T. rubrum* spores, microconidial walls of *Trichophyton mentagrophytes* were described in one study (166). This study reported a microconidial wall thickness up to 400 nm and the presence of a melanin-like pigment. The outer wall consists of a glycoprotein complex (15- 20 nm), the middle electron dense wall represents a rodlet layer embedded in polysaccharides (30-50 nm) and the inner wall consists of glucan and chitin (200-300 nm) (166).

In general, fungal spores differ from somatic cells in the following way (123):

- The wall is thicker. Additional layers include pigments such as melanin.
- The cytoplasm is dense and poorly developed.
- The water content is low and the rate of synthesis of proteins and nucleic acids is low.
- The amount of energy-storage material, such as lipids, glycogen and trehalose is high.

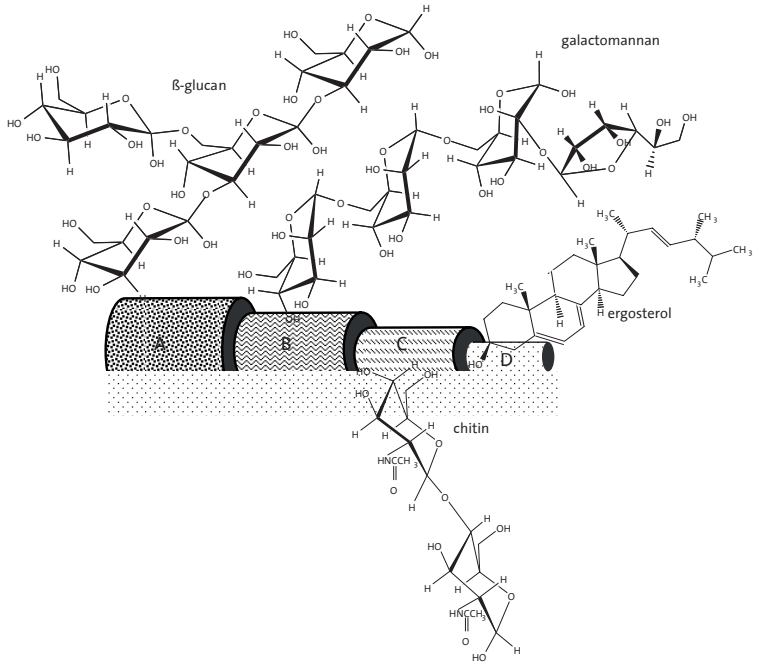

Figure 1.4. Diagrammatic representation of the wall structure of *Trichophyton rubrum*, including the chemical structure of the main units present within the layers that are characteristic for the fungal wall.
(A) Outermost layer of β–1,3-glucans and β-1,6-glucans
(B) Galactomannan, showing α-1,2 en α-1,6 - linked mannopyranose en mannofuranose units
(C) Chitin micro-fibres, embedded within protein
(D) The cell membrane, containing ergosterol.

3 AIM OF THE INVESTIGATIONS AND OUTLINE OF THIS THESIS

The aim of the research described in this thesis was to establish a formulation of porphyrin photosensitizers that, after a single application, cause a complete cure of tinea infection caused by *T. rubrum*.

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This research started with the discovery of the excellent susceptibility of the dermatophyte *T. rubrum* to PDT when using several new types of porphyrin photosensitizers.

A high PDT efficacy was observed for 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, Fig. 1.5). Therefore, the studies described in this thesis mainly focus on these two photosensitizers.

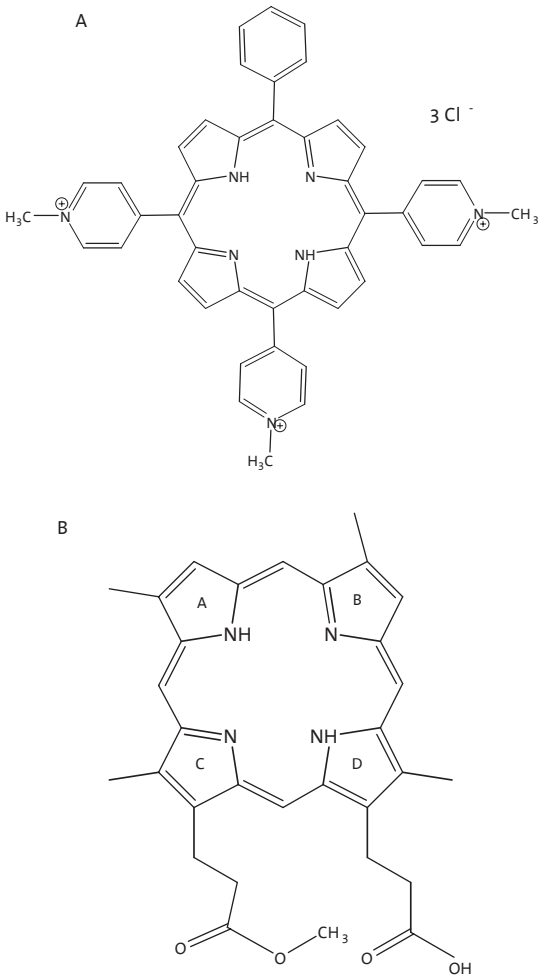

Figure 1.5. Chemical structure of the porphyrin photosensitizer 5,10,15-tris(4-methylpyridinium)-20-phenyl-[21H,23H]-porphine trichloride (Sylsens B, Fig. 1.5A) and deuteroporphyrin monomethylester present as a mixture of 50% with the propyl ester on the D-ring and 50% with this ester on the C-ring (DP mme, Fig. 1.5B).

The results of the *in vitro* studies are described in chapter II and III. During these investigations, the PDT efficacy was tested in suspension cultures with broadband white light (chapter II) and with the red light corresponding to wavelengths between 850 to 870 nm (chapter III). The use of the red light in the photodynamic inactivation of the microconidia and the mycelium was considered to be of great importance as the red light exhibits very good skin penetration and therefore it could find its use also during the research regarding the PDT of onychomycoses. Moreover, *T. rubrum* colonizes both the superficial stratum corneum and the deeper hair follicles, so the good light penetration is of essential importance.

To investigate the proposed photodynamic treatment of *T. rubrum* in experimental setting resembling more closely the clinical situation, a novel *ex vivo* model was developed. This model is described in chapter IV. Of particular importance in this model are the use of human SC as the sole substrate for *T. rubrum* and the adherence of the fungus to this substrate. These are the factors that are known to influence the dermatophyte infection in *in vivo* situations. Additionally, this *ex vivo* model offers the possibility of applying PDT to different fungal growth stages. The model was used to investigate the susceptibility of *T. rubrum* to PDT with the use of two photosensitizers, Sylsens B and DP mme.

To select the optimal formulation for an effective PDT of *T. rubrum,* an additional study was performed to unravel the molecular mechanisms involved in PDT efficacy of both porphyrins. Different physical and chemical aspects of Sylsens B and DP mme concerning their photodynamic action towards *T. rubrum* in different fungal growth stages were investigated in the *ex vivo* situation. This mechanistic study is described in chapter V.

As the currently available drugs do not affect the spores produced by *T rubrum*, in our *in vitro* and *ex vivo* studies, special attention was paid to the PDT effectiveness on the spores produced by *T. rubrum*. The use of PDT could offer an effective solution to this shortcoming of most available treatments.

Chapter VI describes a scanning electron microscopic (SEM) study of morphological changes caused by PDT of *T. rubrum* with the cationic porphyrin Sylsens B. In this study, we focused especially on the effect of a lethal PDT dose on fungal wall morphology and compared it with the effect of the photosensitizer in the dark or with the light dose alone. Disturbances in wall morphology in different fungal growth stages were correlated to the differences in PDT susceptibility, as described in chapter V.

In the past few years, there has been a great improvement in the development of new photosensitizers and their application for medical purposes. However, their safety for medical use is still a matter of investigation. Especially their mutagenic potential is an important issue. Many different test systems for the detection of photochemical genotoxicity have been reported, but most of them have certain limitations. For instance, the well-known and internationally accepted Ames test, although correctly adjusted for light sensitisation experiments, indicated that broad band white light (without added photosensitizer) induced mutagenicity. Another problem is the lack of a reliable positive control for the photomutagenicity test system.

An important qualification for a photomutagenicity test system is that it detects (within one system) the photogenotoxicity caused by either the production of short-lived products, like reactive oxygen species (ROS), or the production of stable photoproducts. The chapter VII of this thesis describes the results of our tests with a newly developed photomutagenicity test system.

For the clinical treatment of tinea it is neither necessary nor desirable for the applied photosensitizer to penetrate the skin. With respect to this important issue, we investigated (described in chapter VIII) the skin penetration of Sylsens B, the best candidate for the PDT of *T. rubrum*. The penetration studies were performed not only using healthy skin but also with stratum corneum disturbed by fungal growth or be pre-treatment with a detergent. Special attention was paid to the porphyrin formulation that displayed the best inhibitory effect on *T. rubrum* grown on human SC.

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Chapter II

PHOTODYNAMIC INACTIVATON OF THE DERMATOPHYTE *TRICHOPHYTON RUBRUM*

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Adapted from: Photochem. Photobiol. 2003May;77(5):556-60

Trichophyton rubrum microconidium with a developing germtube

ABSTRACT

The present study shows that *Trichophyton rubrum* in suspension culture is susceptible to PDT, a completely new application in this area. *T. rubrum* could be effectively killed with the use of the light-activated porphyrins DP mme Sylsens B. The photodynamic efficacy was compared to some other photosensitizers, that are well-known in the field of PDT: the porphyrins, deuteroporphyin DP, hematoporphyrin HP, the drug Photofrin and several phthalocyanines. It was demonstrated that with the use of broadband white light the phthalocyanines and Photofrin displayed a fungistatic effect of about one week, whereas all the porphyrins caused photodynamic killing of the dermatophyte. Sylsens B was the most effective sensitizer and showed no dark toxicity; therefore, in an appropriate formulation, it could be a promising candidate for the treatment of various forms of tinea. For Sylsens B and DP mme, displaying the best results, a concentration dependent uptake by *T. rubrum* was established.

INTRODUCTION

The drugs used today for the treatment of superficial fungal infections are certainly more effective than those available years ago. In the 1960s griseofulvin was introduced (1) as a fungistatic with duration of treatment of 4 weeks up to 18 months (2) while in 1992 a topical treatment with the drug terbinafine (Lamisil) was introduced as a cream that promised to cure a dermatophyte infection after only a few applications (3,4). Recently a sequential pulse therapy with itraconazole (a triazole) and terbinafine (an allylamine) was introduced to treat onychomycosis of fingernails (2,5,6). Allylamines are more effective against dermatophyte infections, but they are more expensive than azoles (2). In general, limitations of the current therapeutic options include: inadequate spectrum of activity, lack of efficacy, multiple drug interactions, inadequate pharmacokinetic profile, excessive costs, recurrence of the infection and duration of treatment (4,7).

The aim of the present study is to determine whether the dermatophyte *Trichophyton rubrum* can be killed with the use of the light-activated porphyrins DP mme and Sylsens B and thus develop a possible clinical application against infections caused by *T. rubrum*. Sylsens B and DP mme are known to be able to kill certain bacteria, Chinese Hamster Ovary cells and the common fruit fly *Drosophila melanogaster* (G.M.T. Smijs, unpublished data). Therefore we selected these compounds to investigate this relatively new application of PDT. Little research has been reported concerning the treatment of fungal infections with the use of light-activated agents. In 1998 the photodynamic effect of different thiophenes on eight strains of dermatophytes with the use of UVA radiation was studied (8). The growth of all tested strains was strongly inhibited by the thiophenes under investigation but a complete inactivation was never detected. Moreover UVA is known to be carcinogenic.

The photodynamic activity of Sylsens B and DP mme towards *T.rubrum* was compared to the photodynamic effect of several photosensitising drugs that have been studied for photodynamic cancer therapy (9), the porphyrins, deuteroporphyin (DP), hematoporphyrin (HP), the drug Photofrin and the phthalocyanines, zinc phthalocyanine (ZnPc), phthalocyanine tetrasulfonate (PcS_4) and aluminium (III) phthalocyanine chloride tetrasulfonate ($AlPcS_4$). For formulae and related names see table 2.1. Photofrin is a commercial product consisting of a complex mixture of monomers, oligomers and aggregates with HP as the starting material.

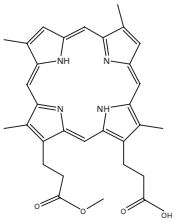
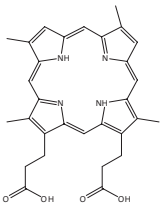
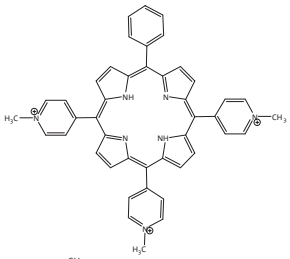
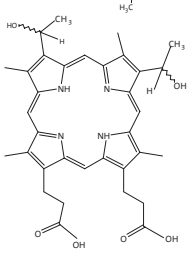
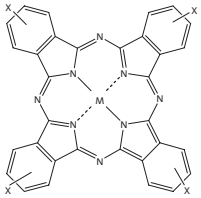
Chemical Structure	*Selected name*	*Abbreviation*
	Deuteroporphyrin monomethylester	DP mme
	Deuteroporphyrin	DP
	5,10,15-tris(4-methylpyridinium)-20-phenyl-[21H,23H]porphine trichloride	Sylsens B
	Hematoporphyrin	HP
	Phthalocyanine	Pc

Table 2.1. Formulae and related names of the porphyrins and phthalocyanines. For ZnPc, M is zinc; for PcS_4, M is hydrogen and X is SO_3H; for $AlPcS_4$, M is aluminium and X is SO_3H.

MATERIALS AND METHODS

Materials

The fungus *T. rubrum* was purchased from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. Cultures were grown on malt extract agar (MEA; Oxoid, Hampshire, UK). Suspension cultures were made in Dulbecco's modified Eagle medium (GibcoBRL, UK) with 2.5% fetal calf serum (GibcoBRL).

Solid cultures were maintained at 28°C, the suspension cultures at room temperature. HP was purchased from Porphyrin Products Inc. (UT, USA); Sylsens B, DP and DP mme 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%). All the phthalocyanines were purchased from Porphyrin Products Inc., and Photofrin was purchased from Lederle Parenterals Inc. (Carolina, USA). Polyethyleneglycol was obtained from Genfarma B.V. (Maarssen, The Netherlands), and 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, HP, Photofrin, ZnPc and $AlPcS_4$; polyethyleneglycol–ethanol–water (3:2:5) for DP; and DP mme and dimethylformamide for PcS_4. Stock solutions of the photosensitizers (2.5 mg/mL solvent) were stored at 4°C for no longer than 1 week.

Photodynamic treatment

Illuminations were performed using a lamp from "MASSIVE" (no. 74900/21), 13 max.500W-230 V-R7s, IP 44. To avoid heating of the samples to be illuminated, the white light produced by the lamp is passed through a 2 cm thick water layer before reaching the samples. Light intensity (30 mW/cm^2) was measured with IL1400A photometer equipped with a SEL033/F/U detector (International Light, Newburyport, MA). Before illumination, the suspension fungal cultures 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 1 hour illumination, the contents of the culture dishes were transferred to 9 cm diameter dishes containing MEA and placed in the incubator at 28°C, and growth was followed during 1 week and quantified by counting the number of inoculates present.

Uptake of photosensitizers

To determine the fungal uptake of the porphyrin photosensitizers Sylsens B and DP mme as a function of time and concentration, suspension cultures (2 mL) were incubated with the photosensitizer at 28°C. Three different concentrations were used with eight different incubation times. After incubation, the suspension culture was centrifuged (Heraeus 3S) for 7 min at 3300 g. The pellet was washed once with medium, three times with Dulbecco's phosphate-buffered saline and finally dissolved in 1 mL of 50% (vol/vol) sulfuric acid. After 24 hours, the concentration of the sensitizer in the sulfuric acid containing dissolved fungus was determined with the use of fluorescence spectroscopy (Perkin–Elmer LS 50B luminescence). The fluorescence emission was measured in the 620–700 nm interval upon excitation at the maximum wavelength of the Soret band of the porphyrin under study. Preliminary experiments for Sylsens B and DP mme showed a linear relationship between fluorescence intensity and porphyrin concentration. A calibration curve was made, and the linear part was used to determine unknown sensitizer concentrations. The values thus obtained were corrected for the recovery, determined separately for Sylsens B and DP mme. To determine the recovery of the sensitizer under study, 2 mL of suspension culture was centrifuged and the pellet was washed as described above. The pellet was dissolved, as described, in sulfuric acid with the addition of predetermined concentrations of the sensitizer, and the fluorescence emission was measured as described above and compared with the fluorescence emission of the actual administered sensitizer concentration in 50% (vol/vol) sulfuric acid. For each sensitizer, five different concentrations were used, and the experiment was repeated six times.

RESULTS

PDT of T. rubrum and dark toxicity

PDT of *T. rubrum* in suspension culture with Sylsens B and DP mme resulted in a complete kill (on the basis of a cutoff at two colonies) of the fungus in almost all experiments. Of the porphyrin derivatives, Sylsens B is by far the most effective. As can be seen in Fig. 2.1A, the photodynamic efficacy of Sylsens B and DP mme above a concentration of 3 µg/mL is analogous to the effect caused by the other porphyrins tested, HP and DP. Below this concentration, Sylsens B displayed a better photodynamic efficacy, whereas the efficacy of DP mme was the same as that of HP; DP gave the

lowest efficacy in this lower concentration range. On comparing with Fig. 2.1B, it can be seen that at lower sensitizer concentrations, PDT merely results in a delay in growth. Only above a concentration of 20 µg/mL was a true fungicidal effect detected for all the porphyrin sensitizers tested. Sylsens B and DP mme displayed this effect even at a lower concentration, namely, 3 µg/mL. For all the porphyrins, it was established that after successful PDT, even after several weeks, no trace of reoccurrence of the fungus on the MEA dishes could be detected. On examining Fig. 2.1C, it however becomes clear that DP mme as well as DP express a dark toxicity at higher concentrations. However, Sylsens B and HP show no dark toxicity under the given circumstances.

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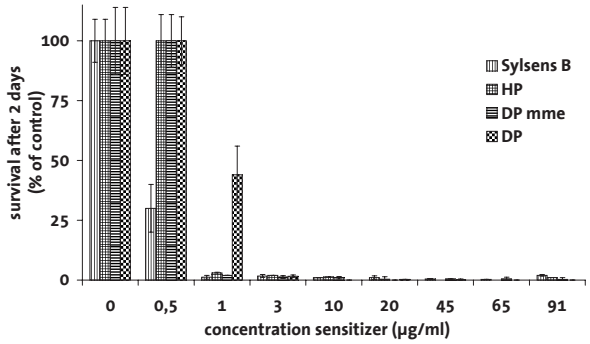
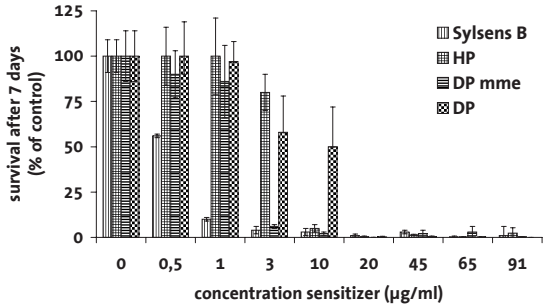


B



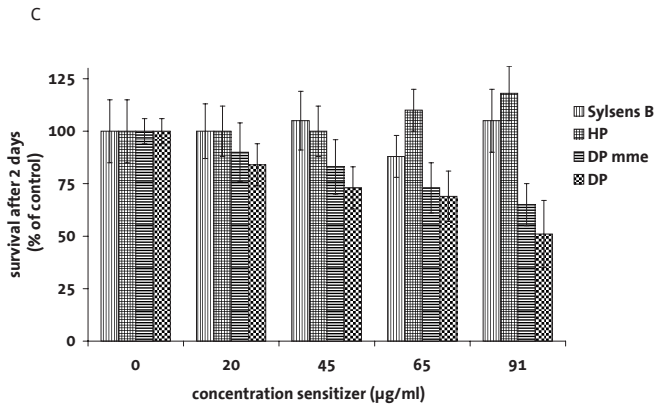


Figure 2.1. Growth of *T. rubrum* on the second (A) and seventh (B) day after light treatment with various porphyrins compared with the growth on the second day after treatment with the porphyrins in the dark (C). Suspension cultures were incubated in the dark for 30 min at 28°C with different concentrations of the porphyrins. After the incubation period, suspension cultures were illuminated with white light (1080 kJ/m^2) or kept in the dark in the presence of the porphyrins. After illumination or dark period, cultures were transferred to MEA dishes and placed in the incubator at 28°C. The survival was measured after 2 (A,C) and 7 (B) days as the number of inoculates present. Untreated cultures containing solvent instead of photosensitizer were considered to have 100% survival. Number of inoculates on control dishes: 175–225 after 2 days and 270–320 after 7 days. The values given are the means of three experiments with the standard deviations.

Figure 2.2A shows the result of PDT of *T. rubrum* using several phthalocyanines and Photofrin. The observed photodynamic efficacy on *T. rubrum* is not as high as that found for the porphyrins (compare with Fig. 2.1A). All the phthalocyanines and Photofrin only induce a growth delay in the first days after the PDT. After 7 days, the fungus grows again as well as it did without PDT, displaying a 100% survival over the whole concentration range used. At day 7, there is no difference between samples subjected to PDT and the blank. So these photosensitizers induce a fungistatic effect that lasts for 7 days. Considering the dark toxicity, only Photofrin shows a positive result when applied at concentrations from 90 µg/mL (see Fig. 2.2B). For the phthalocyanines, no dark toxicity on *T. rubrum* was observed under the conditions used.

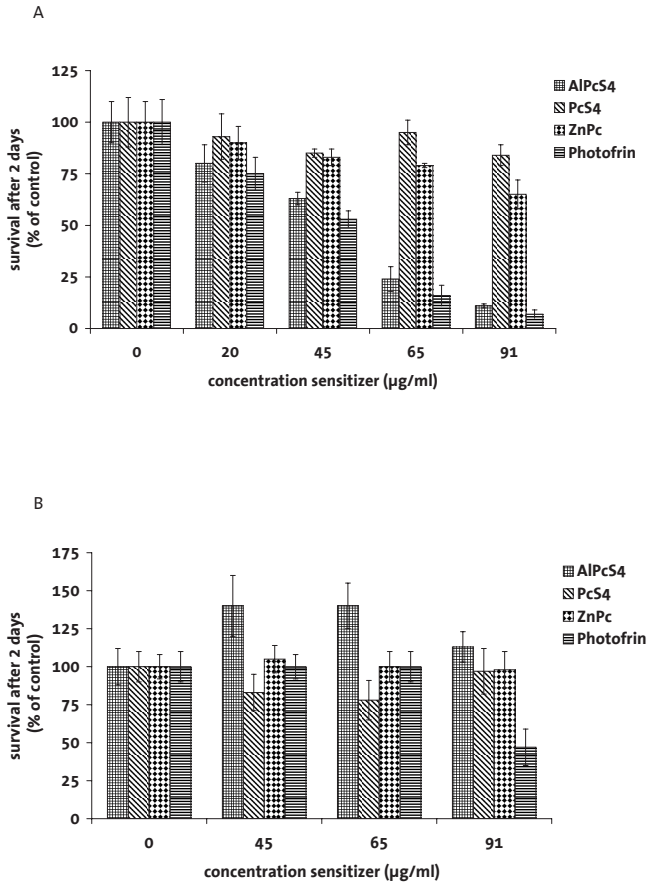


Figure 2.2. Growth of *T. rubrum* on the second day after light (A) or dark (B) treatment with various phthalocyanines and Photofrin. Suspension cultures were incubated in the dark for 30 min at 28°C with different concentrations of the photosensitizers. After the incubation period, suspension cultures were illuminated with white light (1080 kJ/m^2) or kept in the dark in the presence of the photosensitizer. After illumination or dark period, cultures were transferred to MEA dishes and placed in the incubator at 28°C. The survival after 2 days was measured as the number of inoculates present. Untreated cultures containing solvent instead ofphotosensitizer were considered to have 100% survival (number of inoculates: 150–200). The values given are the means of three experiments with the standard deviations.

Uptake of Sylsens B and DP mme

In a first approach to explain the effectiveness of Sylsens B and DP mme, uptake of these two compounds was studied Fig. 2.3 shows the concentration-dependent uptake of the photosensitizers Sylsens B and DP mme by *T. rubrum*, as a function of time. For DP mme, the fluorescence emission was measured at 623 nm (excitation wavelength, 395 nm). For Sylsens B, the emission was measured at 695 nm with an

excitation wavelength of 432 nm. The values shown in the figure are corrected for the recovery (see Materials and Methods), 88% ± 12% for Sylsens B (n = 6) and 84% ± 17% for DP mme (n = 6). Up to an incubation time of 24 h, not much difference is found between the uptake of Sylsens B and DP mme by *T. rubrum*. At longer incubation times, the uptake of the photosensitizer DP mme gives a slightly better result than obtained for Sylsens B. However, significance among the data could only be established for the lowest concentration (16 µg/mL) and highest incubation period and for the highest incubation concentration (55 µg/mL) with 24 h incubation time (Student's *t* -test, *P* = 0.05). Remarkably, no plateau is reached in the uptake of both sensitizers DP mme and Sylsens B. Uptake of the photosensitizer seems to continue.

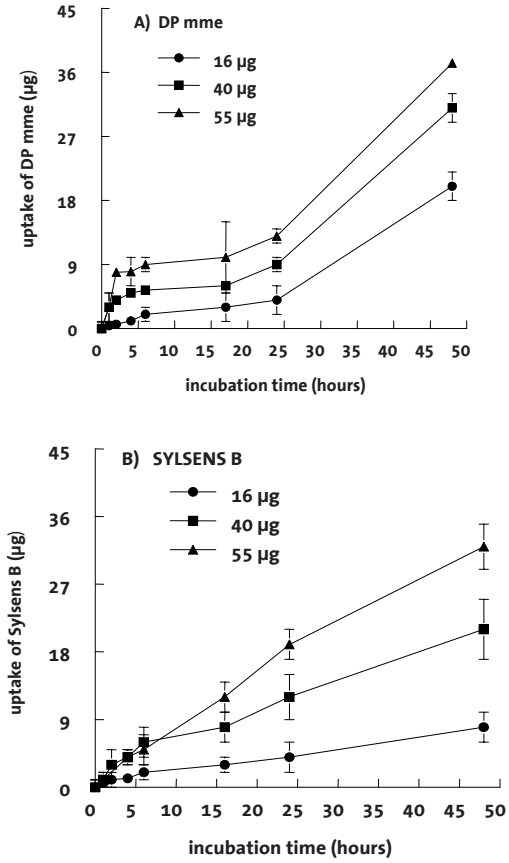


Figure 2.3. Uptake of DP mme (A) and Sylsens B (B) by *T. rubrum*. Incubation temperature: 28°C. For DP mme, the fluorescence emission was read at 623 nm (excitation wavelength, 395 nm). For Sylsens B, the emission was read at 695 nm with an excitation wavelength of 432 nm. Values given are the means of four experiments with the standard deviations. Statistical significance was determined (Student's *t*-test, *P* = 0.05) and was found to be positive for two sets of data points, namely, 55 µg/mL, 24 h and 16 µg/mL, 48 h.

DISCUSSION

Both porphyrins Sylsens B and DP mme were proven to be excellent photosensitizers toward the dermatophyte *T. rubrum*, Sylsens B being the most effective. Both were able to completely prevent the growth of *T. rubrum*, a result that is completely new in the field of PDT. The other tested porphyrins, DP and HP, showed comparable fungicidal effects but at higher concentrations (see Fig.2.1B). For Sylsens B and DP mme, a concentration of 3 µg/mL or higher was sufficient to kill the fungus in most experiments using broadband white light (1080 kJ/m^2). For HP, this concentration appeared to be 10 µg/mL, whereas for DP it was 20 µg/mL. For all the porphyrins, it was established that even several weeks after a successful PDT of the fungus, there was no trace of growth of the fungus on the MEA dishes. This was in contrast with the results obtained with the different phthalocyanines that were tested and Photofrin. They merely displayed a fungistatic effect under the same experimental conditions, whereas all the porphyrins displayed a true fungicidal effect. By 1 week after PDT with the phthalocyanines or Photofrin, the fungus covered the MEA plates completely, a result identical to that obtained with controls, viz. only solvent without sensitizer.

To establish whether the observed photodynamic killing was a true photodynamic effect, we also investigated the dark toxicity of the photosensitizing compounds. We showed that DP and DP mme displayed a dark toxicity under the current experimental conditions at concentrations of 20 µg/mL and higher. Sylsens B and HP showed no dark toxicity in the photodynamic active concentration range. In contrast to these two photosensitizers, with DP mme, dark toxicity most likely contributes to the decrease of the survival as shown in Fig. 2.1. However, the presence of minor dark toxicity does not necessarily have to interfere with a possible future clinical application for PDT of tinea.

On examining Fig. 2.3, it is clear that for Sylsens B and DP mme, there is a similar concentration-dependent uptake into or binding to the fungus. From the current experiments, it cannot be concluded whether the photosensitizers are taken up by the fungus or bound to the outer surface of the fungus. No saturation level could be detected up to 48 h. The difference in uptake kinetics between DP mme and Sylsens B might possibly be explained by the difference in structure of the compounds. Sylsens B is a positively charged, amphiphilic molecule, whereas DP mme is a more hydrophobic molecule.

However, for Sylsens B, the values found for the three different concentrations at the lower incubation times do not significantly differ from each other (Student's *t* -test, $P = 0.05$). This could imply that the uptake curve for Sylsens B has the same shape as that found for DP mme, indicating that there is no difference in uptake kinetics between the two sensitizers. This issue certainly needs to be explored further.

In future research, the photodynamic effect should be examined not only on suspension cultures but also on solid downy colonies grown on agar plates. In a first attempt, we found that when grown as a solid downy colony on an agar plate, *T. rubrum* could not be killed photodynamically in a single treatment with any of the sensitizers described in this article (G. M. T. Smijs, unpublished). At most, a fungistatic effect lasting for no more than 4 days was obtained using short incubation periods. Considering the results of the uptake experiments (see Fig. 2.3), prolonged incubations should be included in further studies. However, it should be taken into account that the growth on an agar plate differs completely from the superficial growth of the fungus on human skin (2). Moreover, PDT *in vitro* could be effective for solid downy colonies using multiple treatments.

The phenomenon of photobleaching (10,11) is a possible source of complications in the clinical application of PDT. However, this is not an issue when using Sylsens B as photosensitizer because this compound does not photobleach (G. M. T. Smijs, unpublished).

Not only the absence of photobleaching and dark toxicity in the photodynamic active concentration range but also the rather low effective concentration supports the idea that Sylsens B is a good candidate for further research into PDT of tinea infections.

Moreover, if the high efficacy that is established *in vitro* can be found again in PDT of tinea infections, this might have certain positive consequences for the costs of the medication.

Because Sylsens B also has absorption peaks in the red part of the spectrum, future research into PDT of the dermatophyte *T. rubrum* with Sylsens B should also include experiments with red light. This can be seen from Fig. 2.4, where the Q-bands of the visible absorption spectrum of the porphyrins used in this study are compared with each other. Because red light has a considerably higher penetration depth in tissue than white light, this property can be particularly useful in treatment of nail infections.

In summary, PDT of tinea infections with Sylsens B as a photosensitizer is a promising entity that deserves further exploration.

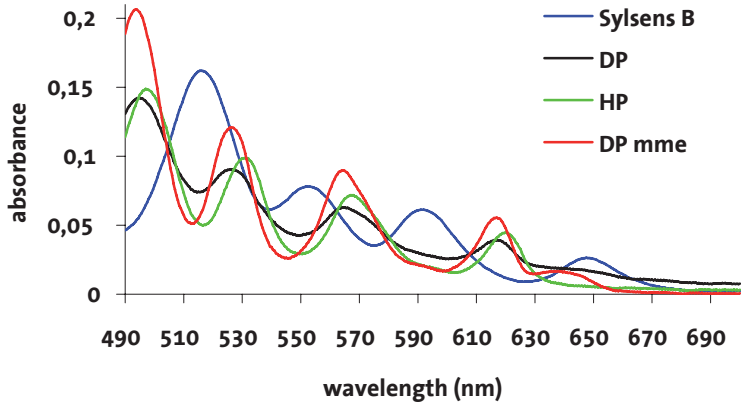


Figure 2.4. Absorption spectra of various porphyrins. The spectra were taken in methanol at a final concentration of 6.5 µg/mL (Shimadzu UV mini 1240). Stock solutions were made in 50 mM sodium phosphate buffer, pH 7.4 (Sylsens B and HP) and polyethyleneglycol–ethanol–water (3:2:5) for DP and DP mme.

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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.

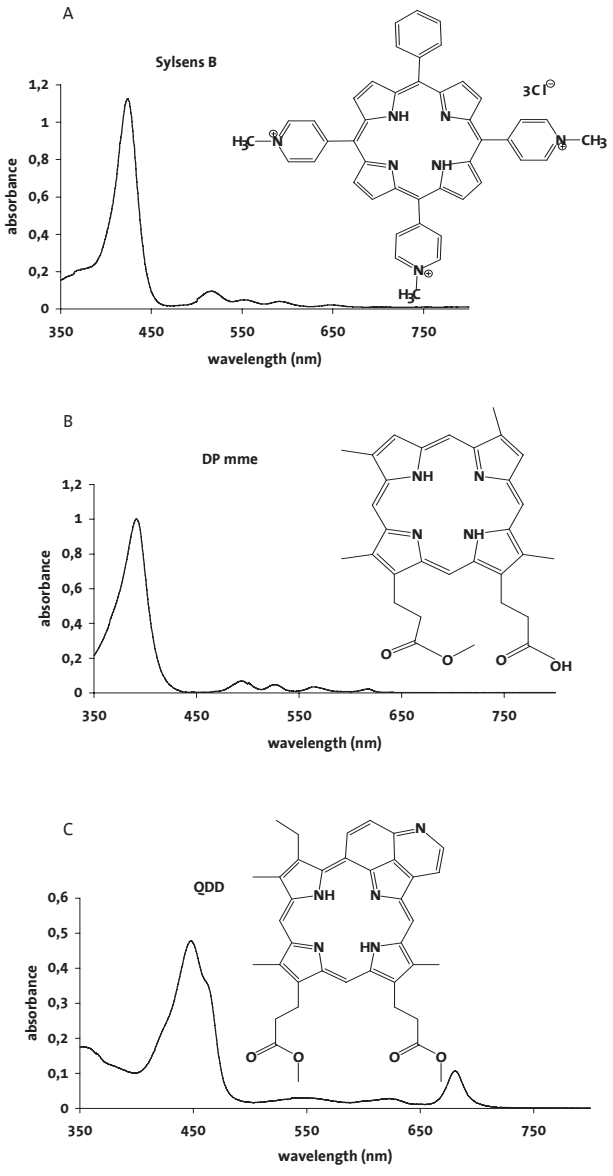

Figure 3.1. Absorbance spectrum in methanol and chemical structure of the porphyrin photosensitizers Sylsens B (A), DP mme (B) and QDD (C). The final concentration in all cases is 5 µM.

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.

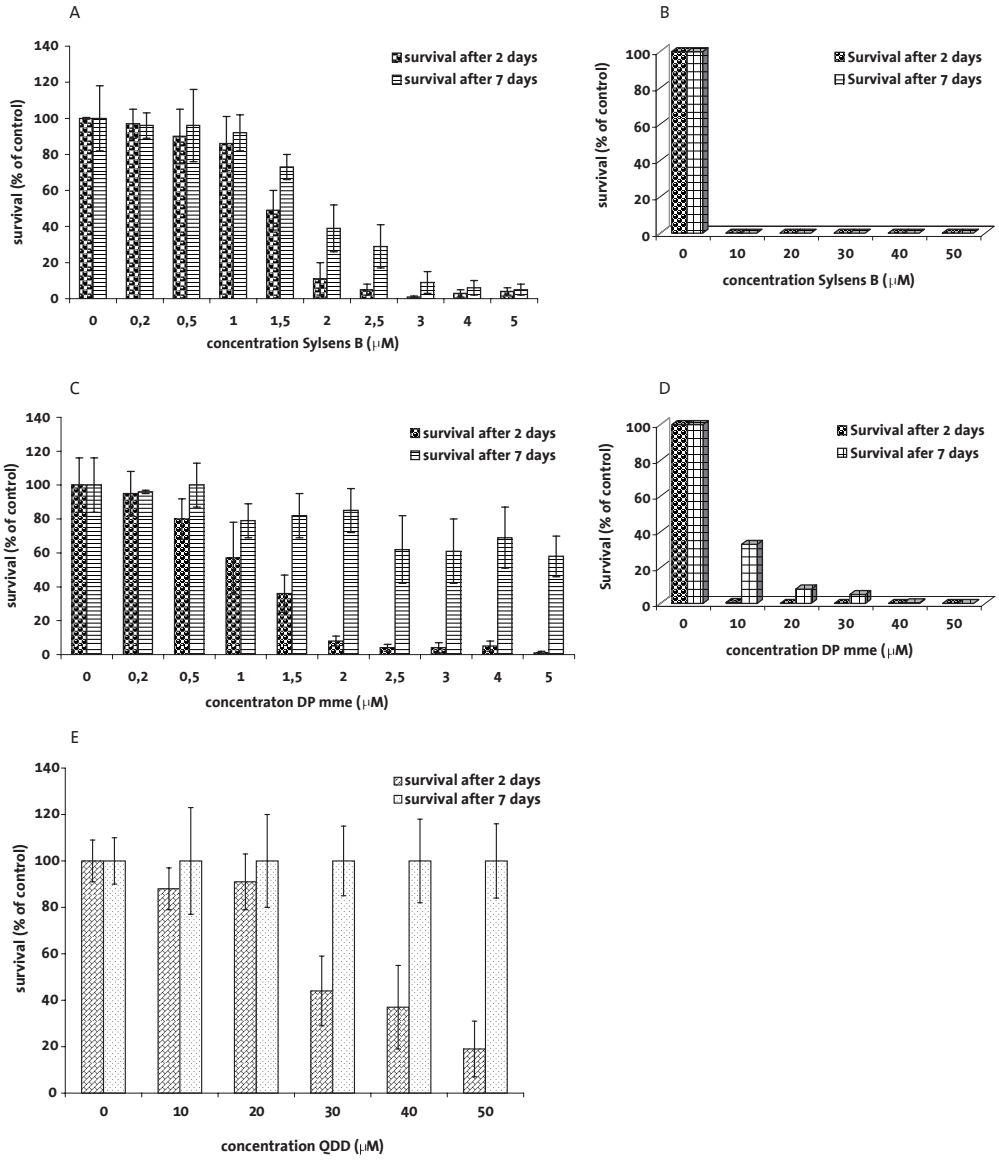

Figure 3.2. Growth of *T. rubrum* after PDT with Sylsens B (A,B), DP mme (C,D) and QDD (E) using red light. Suspension cultures were incubated in the dark for 30 min at 28°C with different concentrations of Sylsens B, DP mme or QDD. After the incubation period, suspension cultures were illuminated with 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.

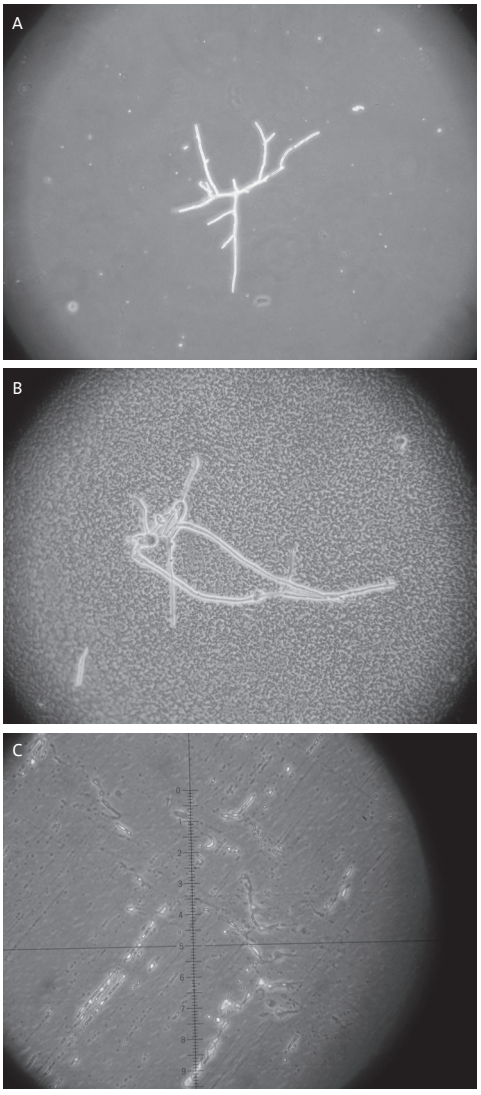

Figure 3.3. Picture taken (Minolta Dimage xi digital camera) from a microscope image (Zeiss Axiovert 25, with 320X magnification) showing hyphen 2.5 weeks after successful PDT with Sylsens B (A) compared with an image of viable hyphen (B) of an untreated culture starting to grow. After 3.5 weeks, the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

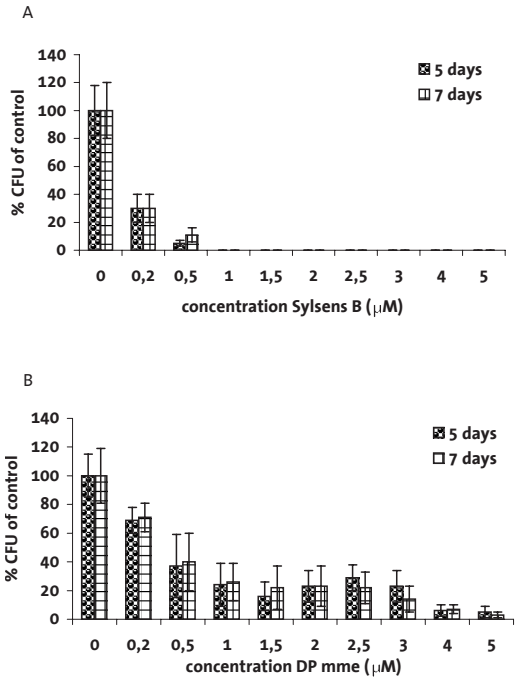

Figure 3.4. Photodynamic effect of Sylsens B (A) and DP mme (B) on the ability of forming cfu from *T. rubrum* microconida using red light. Microconidia suspensions were incubated in the dark for 1 h at 28°C with different concentrations of Sylsens B or DP mme. After the incubation period, spore suspensions 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.

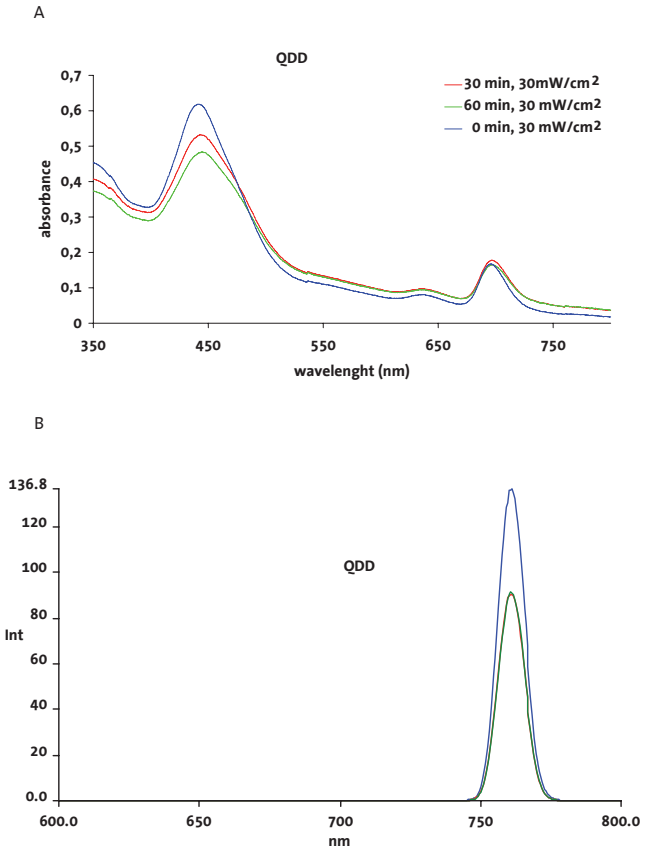

Figure 3.5. Absorption (A) and emission (B) spectra of QDD taken in DPBS before and after illumination with broadband white light (30 mW/cm²). The excitation wavelength was 450 nm and the final concentration of QDD was 15 µM. Blue, 0 min illumination; red, 30 min illumination, 54 J/cm²; green, 60 min illumination, 108 J/cm².

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.

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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Chapter IV

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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Trichophyton rubrum mycelium (72 hours after microconidia inoculation on human stratum corneum)

ABSTRACT

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 PDT, relatively new. Recently, we demonstrated that Sylsens B and DP mme were excellent photosensitizers towards *T. rubrum* when using red light.

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.

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.

INTRODUCTION

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 (1). The dermatophyte *Trichophyton rubrum* causes the most common cutaneous infection in humans, (2,3) which can be very persistent (4). 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 (5). 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 (6,7). 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 (8).

Upon irradiation with light of an appropriate wavelength, photosensitizers can initiate a photochemical reaction resulting in the production of reactive oxygen species, namely singlet oxygen ($^1O_2$) and superoxide anion radical (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 (9). The use of PDT for fungal infections is a new and promising approach (10).

Recently, we have demonstrated that the porphyrins Sylsens B and DP mme are excellent photosensitizers to treat *T. rubrum* in suspension when red light is used to activate them (11). A single PDT *in vitro* was sufficient to achieve a 100% fungicidal effect, using either Sylsens B or 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(12) with two modifications. A 0.01% Tween 80 solution in sterile water was used instead of phosphate-buffered saline and a 7 µm Millipore filter was used instead of a 8 µm 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 µm 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 (10–40 x 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 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 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 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 µm (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 µL 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 (11) 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 Fig. 4.1.

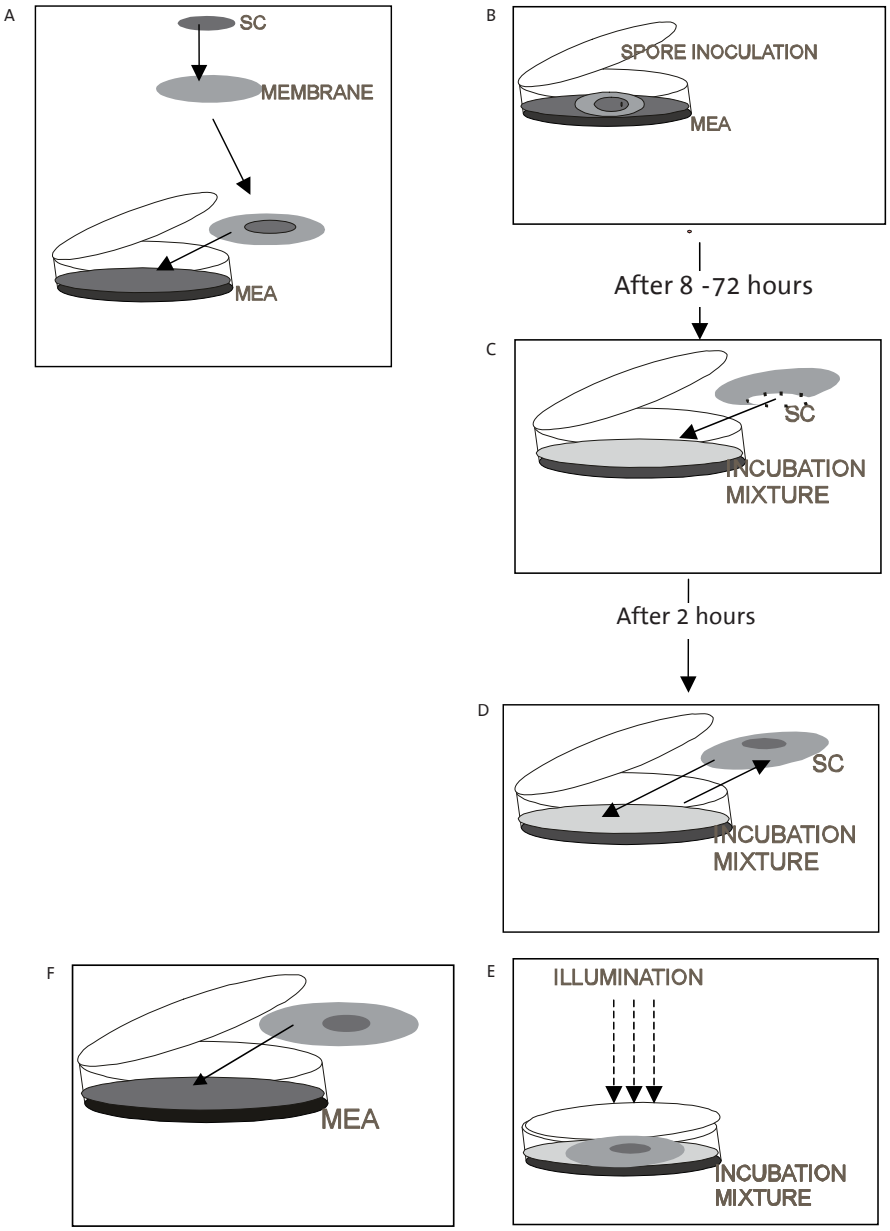

Figure 4.1. Schematic outline of the *ex vivo* model. A polycarbonate membrane filter is placed on a MEA dish and a circular piece of human SC on the membrane (A). The SC is inoculated with a microconidia suspension (B). The membrane is turned upside down and transported with the SC to the incubation 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.

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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 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^2.

Photodynamic treatment

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 µL 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 µL of a photosensitizer solution. Final photosensitizer concentrations were 80 and 160 µM. 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 Fig. 4.1C). 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 (Fig. 4.1D). In all cases, the illumination time was 1 h using a light flux rate of 30 mW/cm^2 (Fig. 4.1E). This corresponds 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 (Fig. 4.1F), 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 CTR 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 x /0.75 Ph2.objective and a HCX PL FLUOTAR 63 x /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 µM Sylsens B in the dark, after an unsuccessful PDT with 80 µM Sylsens B and after a successful PDT with 160 µM 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 4.1. When incubating in DMEM, we found that 8 h after spore inoculation, application of PDT with either 80 or 160 µM 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 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 µM 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.

PDT application: time point, after spore inoculation (h)	Percentage fungicidal effect[b]			
	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)

Table 4.1. 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[a].
The values given are the means of four different experiments (± SEM).
[a] 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/cm²).
[b] A fungicidal effect is defined as the absence of any detectable fungal growth at 8 days after spore inoculation.

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 4.2), the results resembled those obtained with Sylsens B in DMEM (Table 4.1): 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 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 Fig. 4.2 were made at different locations in the same preparation. In this 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 Fig. 4.2A and B).

PDT application: time point, after spore inoculation (h)	***Percentage fungicidal effect[b]***			
	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

Table 4.2. 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[a]
The values given are the means of four different experiments (± SEM).
[a] 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^2).
[b] A fungicidal effect is defined as the absence of any detectable fungal growth at 8 days after spore inoculation.

It can be seen from Fig. 4.2B that under the selected fluorescence microscopic conditions the fungus showed a green coloured autofluorescence. The images shown in Fig. 4.2 (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 (Fig. 4.2C) and the fluorescence images (Fig. 4.2D) 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). Fig. 4.2E (DIC) and Fig. 4.2F (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.
Fig. 4.2 (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 4.2H, 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.

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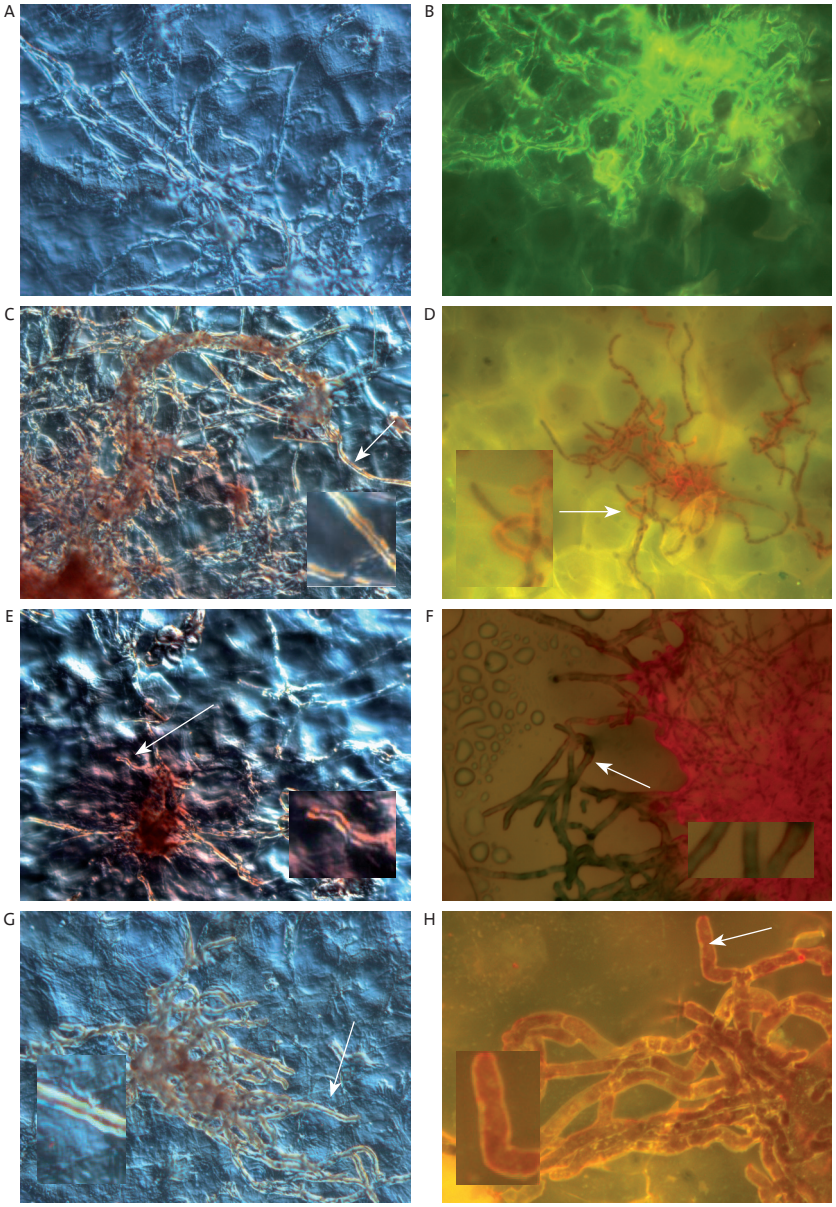

Figure 4.2. *Trichophyton rubrum* on human SC in the *ex vivo* model visualized with differential interference contrast (DIC) and fluorescence microscopy.
Different locations in the same preparation were observed. *T. rubrum* on human SC at 72 h after spore inoculation (A: DIC, 400x and B: fluorescence, 400x). *T. rubrum* on human SC directly after treatment with 160 µM Sylsens B in the dark at 72 h after spore inoculation (C: DIC, 400x and D: fluorescence, 400x). *T. rubrum* on human SC after an unsuccessful PDT with 80 µM Sylsens B, applied at 72 h after spore inoculation (E: DIC, 400x and F: fluorescence, 400x). *T. rubrum* on human SC after a successful PDT with 160 µM Sylsens B, applied at 72 h after spore inoculation (G: DIC, 400x and H: fluorescence, 630x. The arrows point to the localization of Sylsens B (see also the inlays).

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 (13,14). Recently, Seebacher (6) 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 (11). 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 (12,15). 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 (16), 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 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 photodynamic treatments (Fig. 4.2C, D, E and F). However, Fig. 4.2 (G and H) 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 μM Sylsens B and a light dose of 108 J/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 (17). 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 (6,7). 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.

ACKNOWLEDGEMENTS

This work was supported by the Dutch Technology foundation (STW project LKG 6432). We thank Dr. Richard van der Haas from the University of Amsterdam (Laboratory of synthetic Organic Chemistry) and Dr. Rob van der Steen from Buchem Holding BV (Lieren, The Netherlands) for the synthesis of the various porphyrin photosensitizers and for their valuable advice.

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Chapter V

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

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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.

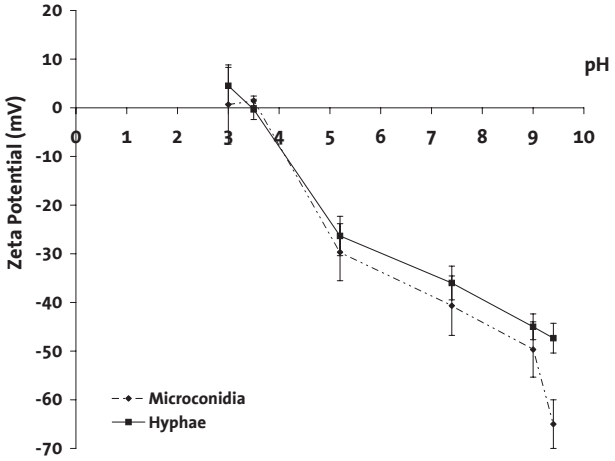

Figure 5.1. Zeta potential of *T. rubrum* microconidia and fungal hyphae, measured as a function of the pH. Values given are the mean values for measurements on three different microconidia and hyphae isolates including the SD.

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.

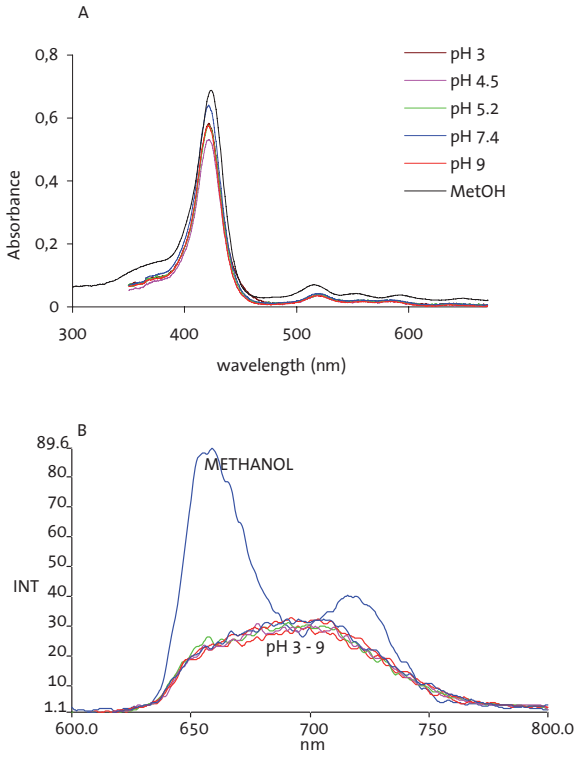

Figure 5.2. Absorption (A) and emission spectra (B) for Sylsens B (2.5 µM), taken in a 5 mM buffer solution of indicated pH or methanol. INT, fluorescence intensity expressed in arbitrary units.

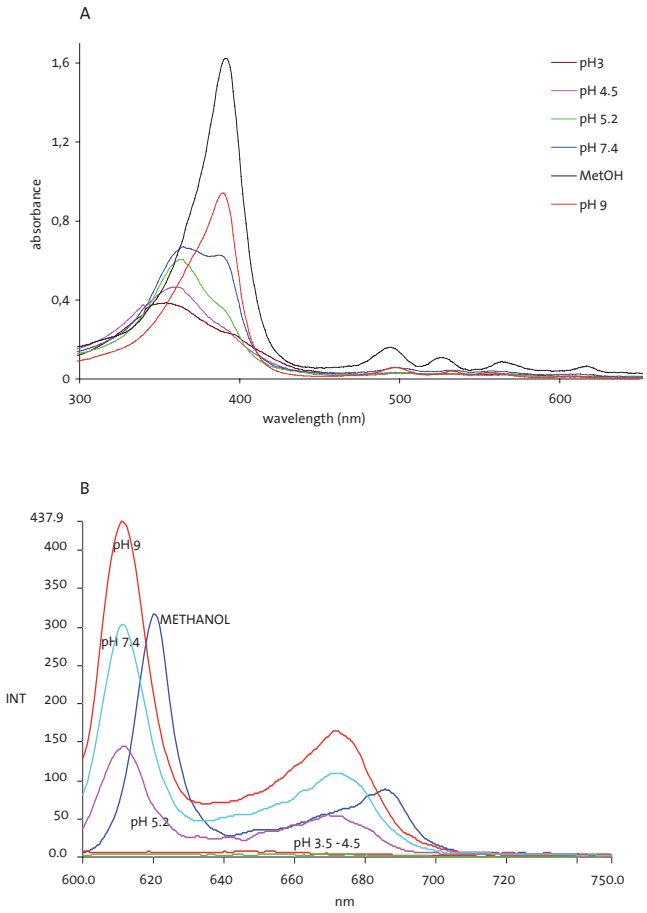

Figure 5.3. Absorption (A) and emission spectra (B) for DP mme (10 µM), taken in a 5 mM buffer solution of indicated pH or methanol. INT, fluorescence intensity expressed in arbitrary units.

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.

regel 1
regel 2
regel 3
regel 4
regel 5
regel 6
regel 7
regel 8
regel 9
regel 10
regel 11
regel 12
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regel 39

	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).

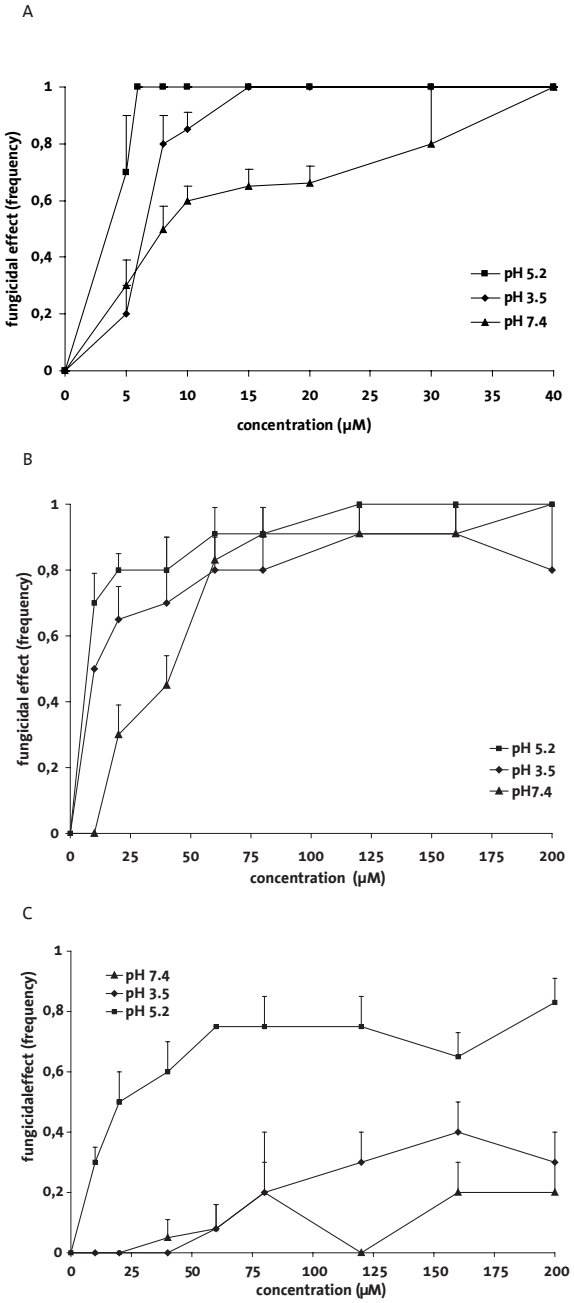

Figure 5.4. Photodynamic efficacy of Sylsens B towards *T. rubrum* tested at 17 (A), 48 (B) and 72 (C) h after spore inoculation on human SC, using a different incubation pH. Application of PDT was performed using the *ex vivo* model with a 2 h incubation period in distilled water of adjusted indicated 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.

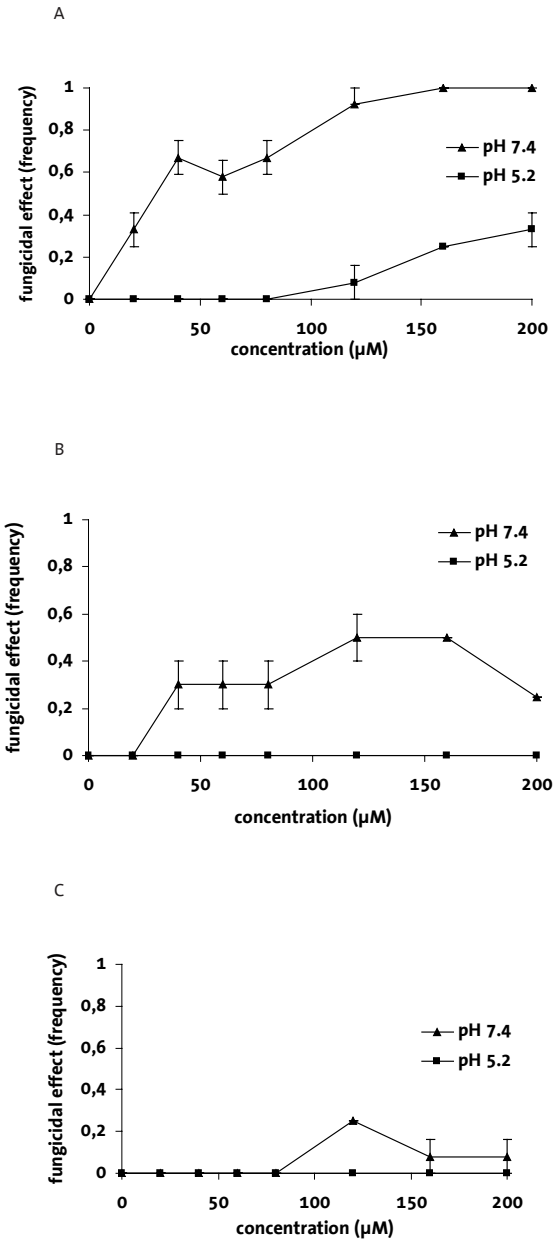

Figure 5.5. Photodynamic efficacy of DP mme towards *T. rubrum* tested at 17 (A), 48 (B) and 72 (C) h after spore inoculation on human SC, using a different incubation pH.
Application of PDT was performed using the *ex vivo* model with a 2 h incubation period in a 5 mM buffer solution of indicated pH (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.

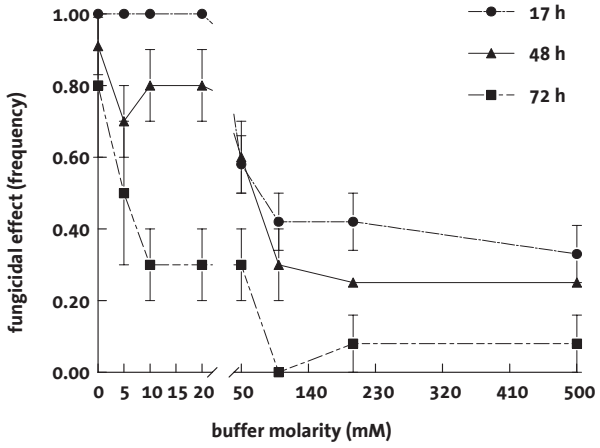

Figure 5.6. Photodynamic efficacy of Sylsens B towards *T. rubrum* tested at 17, 48 and 72 hours after spore inoculation using different molarities of a citric acid/sodium citrate buffer of pH 5.2.
Application of PDT was performed using the *ex vivo* model with a 2 h incubation period in a citric acid/sodium 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).

	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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Chapter VI

MORPHOLOGICAL CHANGES OF THE DERMATOPHYTE *TRICHOPHYTON RUBRUM* AFTER PHOTODYNAMIC TREATMENT: A SCANNING ELECTRON MICROSCOPIC STUDY

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Trichophyton rubrum microconidia, destroyed by photodynamic treatment with Sylsens B and red light

ABSTRACT

Treatment strategies for superficial mycosis caused by the dermatophyte *Trichophyton rubrum* comprise of topical or oral application of antifungal preparations. We have recently discovered a susceptibility of *T. rubrum* to PDT, with Sylsens B as a photosensitizer. The susceptibility depended on the fungal growth stage; the PDT efficacy was higher in microconidia when compared to mycelium.

The aim of this study was to investigate the morphological changes caused by a lethal PDT dose to *T. rubrum* grown on isolated human stratum corneum with the use of scanning electron microscopy. Corresponding dark treatment and a light treatment without photosensitizer were used as control. Sub-lethal PDT was also included. The morphologic changes were followed at various time points after the treatment of different fungal growth stages.

Normal fungal growth was characterized by a fibre-like appearance of the surface of the hyphae and microconidia with the exception of the hyphal tips in full mycelium and the microconidia shortly after attachment to the stratum corneum. Here, densely packed globular structures were observed. The light dose (108 J/cm^2) in the absence of Sylsens B or the application of the photosensitizer in the absence of light caused reversible fungal wall deformations and bulge formation. However, after a lethal PDT, a sequence of severe disruptions and deformations of both microconidia and mycelium was observed leading to extrusion of cell material and emptied fungal elements. In case of a non-lethal PDT, fungal re-growth started on the remnants of the treated mycelium.

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INTRODUCTION

Superficial dermatomycosis caused by the dermatophyte *Trichophyton rubrum* is one of the most common human skin infections worldwide (1-4). The treatment often comprises the use of an antifungal drug in either a topical or an oral application (5-7) or a combination of both (8) .The frequently used drugs can be divided into three different groups, the polyenes, the azols and the allylamines (9,10). Apart from these groups griseofulvin (10) and cyclopiroxolamine (11) are occasionally used. Furthermore, efforts have been made to use PDT for dermatophyte infections (12-14). We have recently demonstrated that *T. rubrum* is also susceptible to PDT with the synthetic porphyrin photosensitizer, Sylsens B, both *in vitro* (15) and *ex vivo* (16). PDT refers to the use of light-activated agents, called photosensitizers, in combination with light of an appropriate wavelength and molecular oxygen (17). This photochemical reaction results in the production of reactive oxygen species, namely singlet oxygen ($^1O_2$) and superoxide anion radial (O_2^-). As a consequence, the photodynamic effect can cause the injury of cells and different kind of pathogens if they are in close proximity to the photosensitizer (18), leading to an effective treatment for localised infections (19). It is generally agreed that $^1O_2$ is the key agent responsible for the cellular damage during PDT. However this reactive oxygen has a short life time and hence its diffusion distance is very small (< 200 nm) (20). A selective, tight binding of the photosensitizer to the target microorganism is thought to be necessary for PDT effectiveness (21-23). Several studies concerning the action mechanisms of antifungals have been published (10,24-27). Some of the morphological studies showed degenerative changes of fungal mycelium and spores (10,28,29). Some of these investigations also included *T. rubrum* (30-32). However, there have been few reports describing morphological changes caused by PDT to microbes in general (33,34) and to *T. rubrum*, in particular.

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

In addition, we visualized the growth of this dermatophyte after sub-lethal PDT. The application of the different treatments was performed on a novel *ex vivo* model developed by us (16). Since our previous studies revealed that the susceptibility of *T. rubrum* to PDT with Sylsens B depended on the fungal growth stage (23), it was decided to visualize the morphological changes at various growth stages and at different time points after the treatment. For the different treatments the selected fungal growth stages corresponded to 8, 48 and 72 hours after spore inoculation on isolated human SC present in the *ex vivo* model (16). Under the given conditions, the 8-hour growth stage was characterized by germinating microconidia and the absence of fungal hyphae. At 48 hours after spore inoculation, hyphae growth started, while the 72-hour growth stage was represented by full mycelium.

MATERIALS AND METHODS

Materials

The fungus *T. rubrum* was purchased from the Centraalbureau voor Schimmelcultures (CBS, strain no: 304.60), Utrecht, The Netherlands. For the preparation of a microconidia suspension *T. rubrum* cultures were grown on Sabouraud Dextrose Agar (Sigma-Aldrich Chemie, Germany) at room temperature.

Sylsens B (mol wt: 769.16 g/mol) was synthesized by Buchem Holding BV (Lieren, The Netherlands; purity was more than 99 % as determined with NMR). Glutaraldehyde and OsO_4 were purchased from Electron Microscopy Sciences (Hatfield, Great-Britain), the cacodylate buffer from Sigma (Sigma-Aldrich Chemie, Germany), while all other chemicals were purchased from J.T.Baker (Deventer, The Netherlands). Stock solutions of 6 mM Sylsens B were prepared and stored at 4°C for no longer than one week.

Preparation microconidia suspension

The protocol for obtaining a suspension of microconidia produced by *T. rubrum* grown on Sabouraud Dextrose Agar was based on a method as described previously (16,35). The obtained microconidia suspensions (10– 40 x 10^6 colony forming units (cfu) /mL) were stored in liquid nitrogen for no longer than 6 months. Counting the number cfu on malt extract agar (MEA) dishes was used as a viability check.

Preparation of the human SC

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

Light source

Illuminations were performed with a lamp from "MASSIVE" (no.74900/21), 1× max.500W-230 V-R7s, IP 44. To avoid heating of the samples during illumination, a 5 cm water filter was used to absorb infrared radiation. Light intensity was measured with an IL1400A photometer equipped with a SEL033/F/U detector (International Light, Newburyport, MA, USA). A red cut-off filter at 600 nm was used to obtain the red 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 irradiance at the sample of the infected human SC was 30 mW/cm^2.

The ex vivo model and photodynamic treatment

For the photodynamic, dark and light treatment, a *T. rubrum* microconidia suspension (45 to 450 cfu) was inoculated on human SC in the *ex-vivo* model as described before (16). Before the irradiation, the pieces of SC, with the microconidia inoculates were kept in an incubator at 28°C. The different treatments were performed in a water incubation medium of pH 5.2 supplemented with Sylsens B (with the exception of the light treatments). As described in the *ex-vivo* model, the membrane filter containing the SC and the treated fungus was transferred after every treatment to a malt extract agar (MEA) dish, and placed in an incubator at 28°C (16).

The fungus was subjected to the treatments at 8, 48 and 72 hours after spore inoculation. In case of PDT or dark treatment carried out 8 hours after spore inoculation, 20 µM Sylsens B was used. For PDT or dark controls performed at 48 and 72 hours after spore inoculation, 200 µM Sylsens B was used. Previous studies reported that PDT applied at 72 hours after spore inoculation with 200 µM Sylsens B resulted in 80 to 90 percent of the cases in a complete fungal inactivation. In the remaining cases we observed one

or two fungal colonies appearing at 5 days after the treatment (23). The described situation was defined as sub-lethal PDT. A light dose of 108 J/cm^2 was applied during all photodynamic and light treatments.

Scanning electron microscopy

In order to investigate structural alterations of *T. rubrum* following the different treatments, scanning electron microscopy was applied to visualise the (treated) fungus located on the sheets of human SC. Untreated *T. rubrum* inoculates on human SC, in different growth stages, were used as control. The samples were examined at 1, 20 and 40 hours after the treatment or at 8 days after spore inoculation. A JEOL JSM-6700F field emission SEM (Tokyo, Japan) was used at an acceleration voltage of 5 kV.
The SC sheets were fixed overnight at 4°C in 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) and post-fixed on ice during 1 hour in 1 % OsO_4 (in 0.1 M sodium cacodylate buffer of pH 7.2). Subsequently, the samples were rinsed (3 times) with PBS and dehydrated in increasing concentrations of ethanol (50-100%). The samples were than critical point dried with CO_2 using a Baltec CPD 030 (Balzers, Lichtenstein) and coated with gold-palladium using an Emitech K650X sputter coater (Ashford, England). Every experiment within a chosen growth stage and treatment was repeated at least 4 times (4 duplicates within every experiment) and representative images were selected (from approximately 150 to 200 for every condition and corresponding growth stage). Results were summarized and scored by three persons (one of them blinded).

RESULTS

The results of the PDT treated samples, dark and light controls, at different time points after spore inoculation, are presented and compared to normal fungal growth. The score results of the structural fungal wall alterations after the different treatment modalities are summarized in table 6.1.

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A

Treatment	Minor wall Deformations		Bulge formation		Smooth wall appearance (loss of fibre-like Structure)		Extrusion of Cell material		Flattening	
	I[2]	II[3]	I	II	I	II	I	II	I	II
PDT	100	100	45	23	0	0	8	73	16	93
Dark control	100	6	18	Sporadically	0	0	3	3	3	3
Light control	74	8	Sporadically	Sporadically	0	0	0	0	0	0
none	0	6	Sporadically	Sporadically	0	0	0	0	0	0

B

Treatment	Minor wall Deformations		Bulge formation		Smooth wall appearance (loss of fibre-like Structure)		Extrusion of Cell material		Flattening	
	I[2]	II[3]	I	II	I	II	I	II	I	II
PDT	100	100	30	28	45	88	4	65	13	87
Dark control	100	10	12	Sporadically	0	0	2	3	3	3
Light control	100	12	Sporadically	Sporadically	0	0	0	0	0	0
none	5	10	Sporadically	Sporadically	0	0	0	0	0	0

C

Treatment	Minor wall Deformations		Bulge formation		Smooth wall appearance (loss of fibre-like Structure)		Extrusion of Cell material		Flattening	
	I[2]	II[3]	I	II	I	II	I	II	I	II
PDT	100	100	30	21	43	86	5	57	10	95
Dark control	100	9	11	Sporadically	0	2	1	1	1	0
Light control	100	10	Sporadically	Sporadically	0	0	0	0	0	0
none	3	10	Sporadically	Sporadically	0	0	0	0	0	0

Table 6.1. Structural fungal wall alterations[1] observed as a result of PDT, light only treatment (108 J/cm^2) and dark treatment with the photosensitizer Sylsens B alone of *T. rubrum* applied at 8 (A), 48 (B) and 72 (C) hours after spore inoculation.

[1] The number of observed alterations is expressed as the percentage of the number of fungal elements scored in total. In total 60 to 80 fungal elements from different preparations were scored.

[2] Eight hours after treatment.

[3] Eight days after spore inoculation.

Eight hours after microconidia inoculation

SEM images of the *T. rubrum* growth stage corresponding to eight hours after spore inoculation are shown in figures 6.1 and 6.2. At this time point, the microconidia were attached to the SC and the germination process had started (Fig. 6.1). Application of PDT with 20 μM Sylsens B [known to cause complete fungal inactivation (23)] caused wall deformations and leakage of internal cell material shortly after the treatment (Fig. 6.2-A1 and 6.2-A2). Seven days later, only the remains of the successfully treated germlins could be observed (Fig. 6.2-A3). Control dark treatment with the same Sylsens B concentration resulted in minor wall deformations such as bulge formation (Fig. 6.2-B). Treatment with light alone (108 J/cm^2) at 8 hours after spore inoculation resulted in minor wall deformations, such as indented fungal wall (Fig. 6.2-C). Later fixation times showed fungal recovery in the case of control dark or light treatment. This indicates that the observed changes in the fungal wall appearance are reversible. Figure 6.3 provides a typical example of fungal growth at eight days after spore inoculation under *ex vivo* conditions in the absence of PDT. There is full and intense mycelium growth of *T. rubrum*. A similar growth was observed after a dark or light treatment only, indicating again that the morphological alterations due to dark or light treatment (observed shortly after the treatment) are not permanent, and normal fungal growth was observed at eight days after spore inoculation.

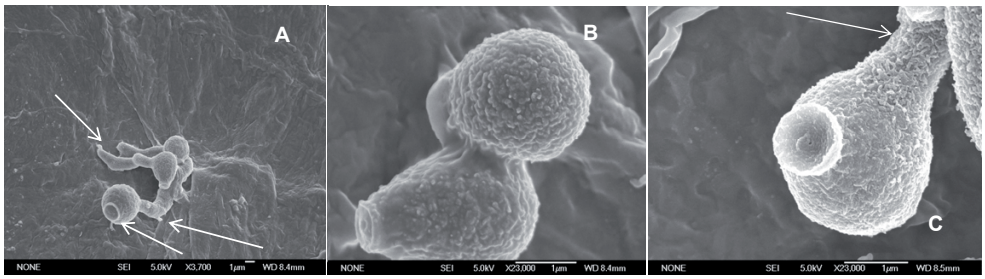

Figure 6.1. SEM images showing *T. rubrum* microconidia 8 hours after spore inoculation on human SC. In figure A a number of microconidia, just germinated, are depicted. Clearly shown are the adherences of the microconidium and germ tube to the SC can (white arrows). In figure B, the regular globular structure of the microconidia wall is noticed, while the wall structure in C (after microconidium germination) has a more fibre-like appearance. The white arrow points to the germ tube.

Forty-eight hours after microconidia inoculation

The figures 6.4 and 6.5 cover the results obtained at this fungal growth stage. At 48 hours after spore inoculation, hyphae proliferation was in full development. In Fig.

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6.4 we focused the attention on the appearance of the outer wall of the fungal hypae in this proliferation stage. The structure of the hyphal wall resembled that of a fibre mesh, resulting in a rough appearance. One and twenty hours after the lethal PDT, bulge formation and a ruptured wall were observed (Fig. 6.5-A1 and A2) as well as small smooth appearing hyphal wall areas (inlay in Fig. 6.5-A2). Here, the loss of the wall fibres had started. At a later time point (forty hours after the treatment) and in extreme cases the fibre-rich structure disappeared, rendering a complete smooth appearance of the outer wall (Fig. 6.5-A3). Eventually, after the successful PDT (8 days after the original spore inoculation), flattened and dented hyphae were observed (Fig. 6.5-$A4_a$ and 6.5-$A4_b$).

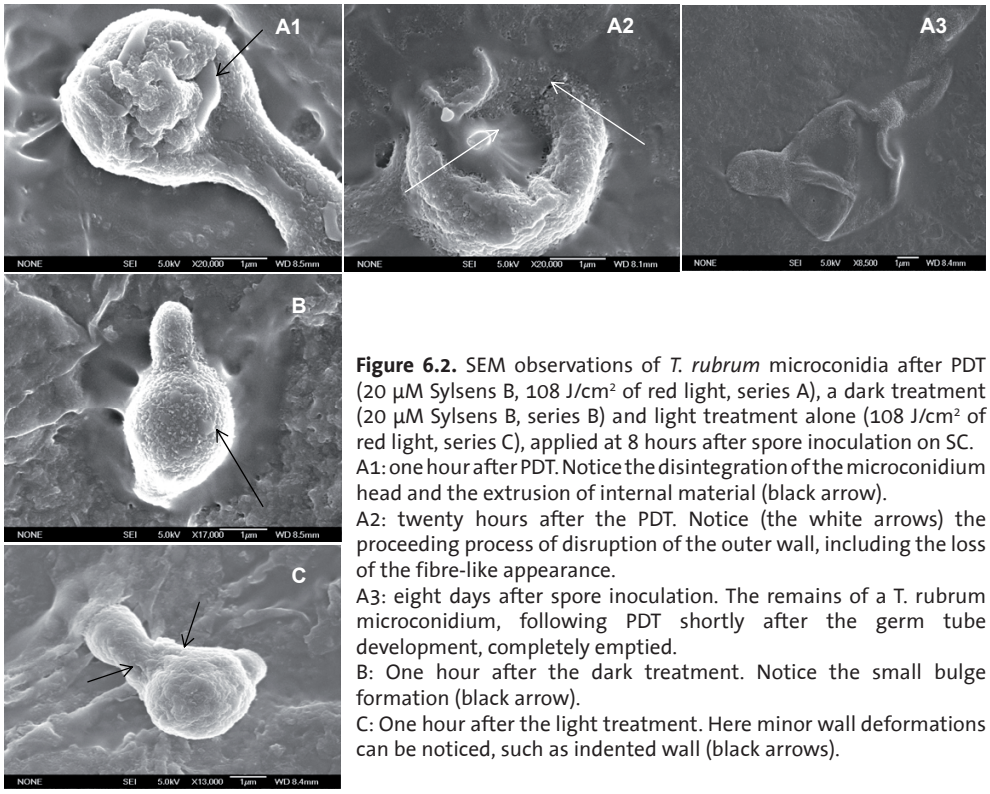

Figure 6.2. SEM observations of *T. rubrum* microconidia after PDT (20 µM Sylsens B, 108 J/cm² of red light, series A), a dark treatment (20 µM Sylsens B, series B) and light treatment alone (108 J/cm² of red light, series C), applied at 8 hours after spore inoculation on SC.
A1: one hour after PDT. Notice the disintegration of the microconidium head and the extrusion of internal material (black arrow).
A2: twenty hours after the PDT. Notice (the white arrows) the proceeding process of disruption of the outer wall, including the loss of the fibre-like appearance.
A3: eight days after spore inoculation. The remains of a T. rubrum microconidium, following PDT shortly after the germ tube development, completely emptied.
B: One hour after the dark treatment. Notice the small bulge formation (black arrow).
C: One hour after the light treatment. Here minor wall deformations can be noticed, such as indented wall (black arrows).

Occasionally, we observed a severely damaged fungus after treatment with 200 µM Sylsens B in the dark (Fig. 6.5-B). However, in most cases, dark treatment resulted in similar (but less severe) deformations, while *T. rubrum* treated with 108 J/cm² of red light alone had an almost normal appearance (Fig. 6.5-C).

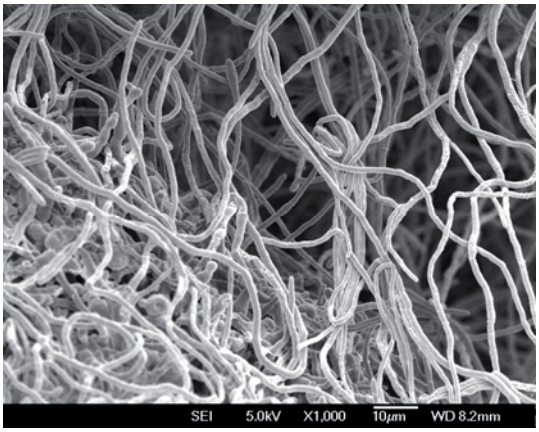

Figure 6.3. A SEM visualization of *T. rubrum* growing on human SC under normal *ex vivo* model conditions, eight days after spore inoculation.

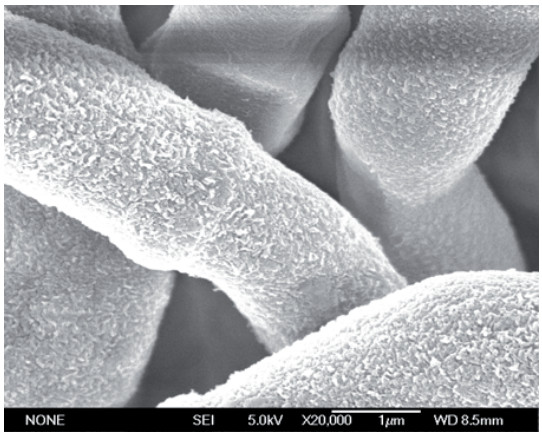

Figure 6.4. A SEM image showing the normal appearance of *T. rubrum* hyphae, displaying a fibre mesh, at 48 hours after inoculation of a microconidia suspension on human SC. Notice the rough surface of the fungal hyphae.

Seventy-two hours after microconidia inoculation

The growth of *T. rubrum* on SC in the *ex vivo* model at 72 hours after spore inoculation can be seen clearly in Fig. 6.6. In 6.6-A the full mycelium is clearly shown. Sporulation of the dermatophyte had occurred under the given *ex vivo* conditions, within three days (see Fig. 6.6-B and 6.6-C). Of particular interest is the difference in fungal wall structure in the middle of hyphae compared to the hyphal tips (Fig. 6.6-D and 6.6-E).

The tips were more densely packed with material of globular appearance, a difference that could not be observed in the other growth stages. The morphology of the hyphal wall in the branching areas is shown in Fig. 6.6-F. Close inspection of the branching point showed a densely packed, globular structure.

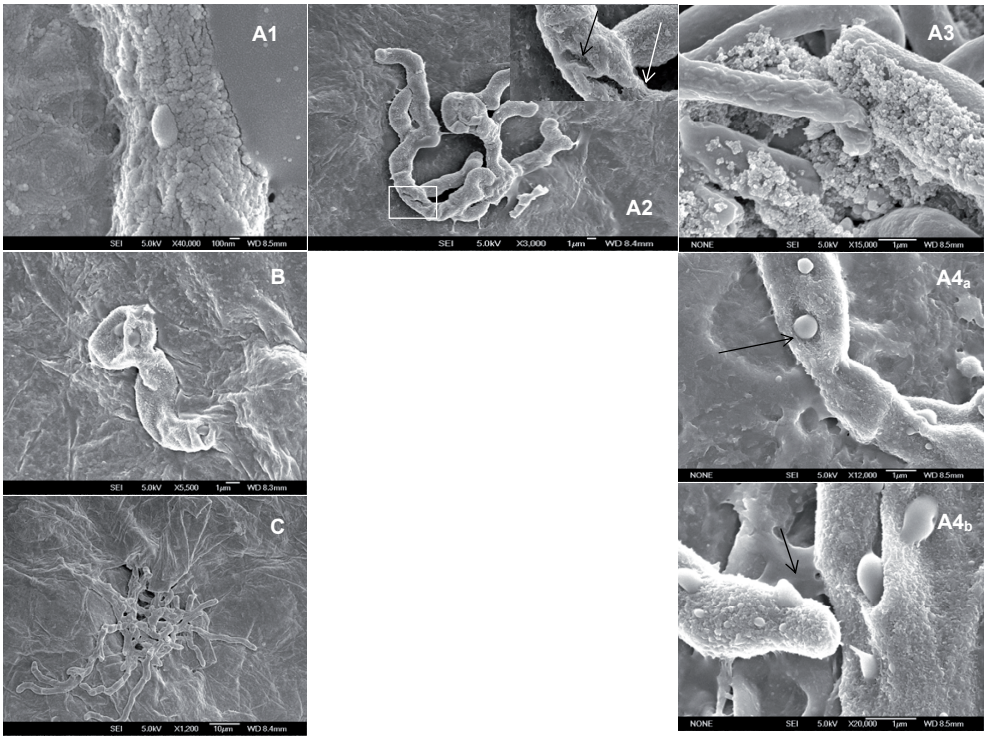

Figure 6.5. SEM images of *T. rubrum* after lethal PDT (200 µM Sylsens B, 108 J/cm² of red light, series A), dark treatment (200 µM Sylsens B, series B) and light treatment alone (108 J/cm² of red light, series C), applied at 48 hours after spore inoculation on SC.
A1: a hyphae close-up, one hour after the PDT. Notice the bulge formation in the middle.
A2: twenty hours after PDT. As can be seen in the inlay (magnification: 20.000 X), a complete rupture of the hyphae wall occurred (black arrow). Notice the disappearance of the rough fibre-rich structure rendering a smooth appearing wall surface (white arrow in the inset).
A3: forty hours after the PDT. We observed a complete loss of the wall fibre-mesh material rendering smooth, naked, hyphae elements.
A4: eight days after spore inoculation. At this time point after PDT the fungal hyphae show a flattened appearance and areas of bulge formation (black arrow in $A4_a$) and leakage (black arrow in $A4_b$) are observed.
B: twenty hours after the dark treatment, showing a severely damaged (wall flattening, loss of structure and bulge formation) fungal element.
C: one hour after the light treatment.

The application of a lethal PDT dose at 72 hours after spore inoculation resulted not only in fibreless fungal hyphae (one hour after the treatment, Fig. 6.7-A1$_a$ and 6.7-A1$_b$), flattened and emptied mycelium structures (Fig. 6.7-A2) twenty hours after the treatment, but also in other time related morphological changes. Worth mentioning is the abundant bulge formation, observed 8 days after the original spore inoculation (5 days after PDT), on the already smooth appearing hyphae (Fig. 6.7-A3). As a result of the PDT, the hyphae were deprived of their fibre-like, grainy wall material giving them a smooth appearance.

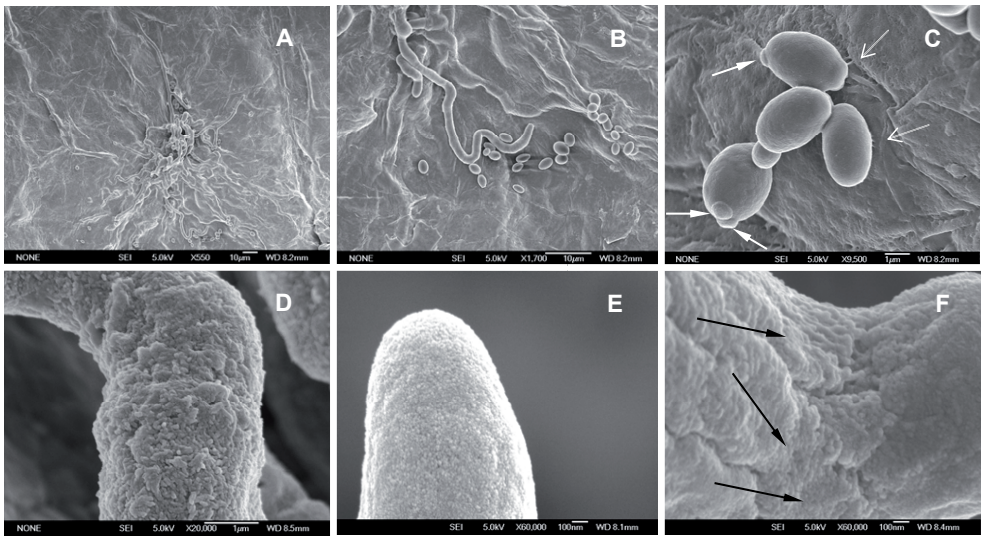

Figure 6.6. Different SEM images all showing *T. rubrum* growing on human SC 72 hours after spore inoculation. In A full mycelium growth can be seen. Notice in B, and in close-up in C, the formation of new microconidia within three days after spore inoculation. In C the closed white arrows point to the places of germ tube formation and the open arrows point to the tender points of attachment of the microconidia to the SC. In figure D a close-up is shown of the wall surface in the middle of a fungal hyphae and in E of a growing tip (notice the closely packed globular appearance here). In F a close up was taken of new hyphal branching. The arrows indicate areas displaying a more globular structure.

The dark treatment (Fig. 6.7-B) caused some wall deformations, while for the light treatment hardly any changes could be observed (Fig. 6.7-C). From both images in Fig. 6.8, it is clear that in the case of an ineffective PDT, fungal re-growth started on the remains of the fungal mycelium. As can be observed during a close inspection, re-growth appeared to start not only on the growing tips but also in other areas of the "old" fungal hyphae.

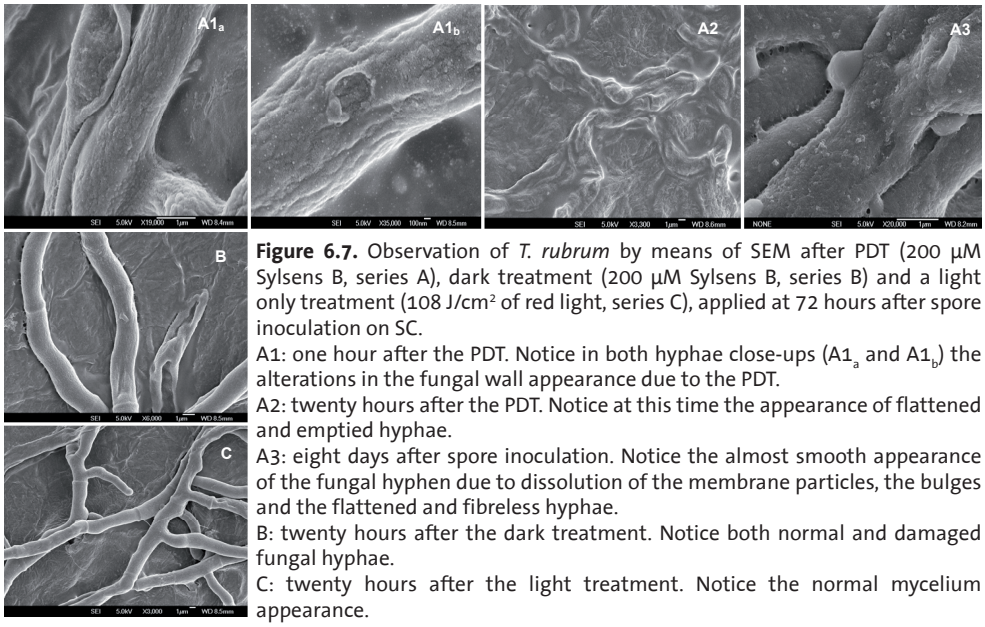

Figure 6.7. Observation of *T. rubrum* by means of SEM after PDT (200 µM Sylsens B, series A), dark treatment (200 µM Sylsens B, series B) and a light only treatment (108 J/cm² of red light, series C), applied at 72 hours after spore inoculation on SC.
A1: one hour after the PDT. Notice in both hyphae close-ups ($A1_a$ and $A1_b$) the alterations in the fungal wall appearance due to the PDT.
A2: twenty hours after the PDT. Notice at this time the appearance of flattened and emptied hyphae.
A3: eight days after spore inoculation. Notice the almost smooth appearance of the fungal hyphen due to dissolution of the membrane particles, the bulges and the flattened and fibreless hyphae.
B: twenty hours after the dark treatment. Notice both normal and damaged fungal hyphae.
C: twenty hours after the light treatment. Notice the normal mycelium appearance.

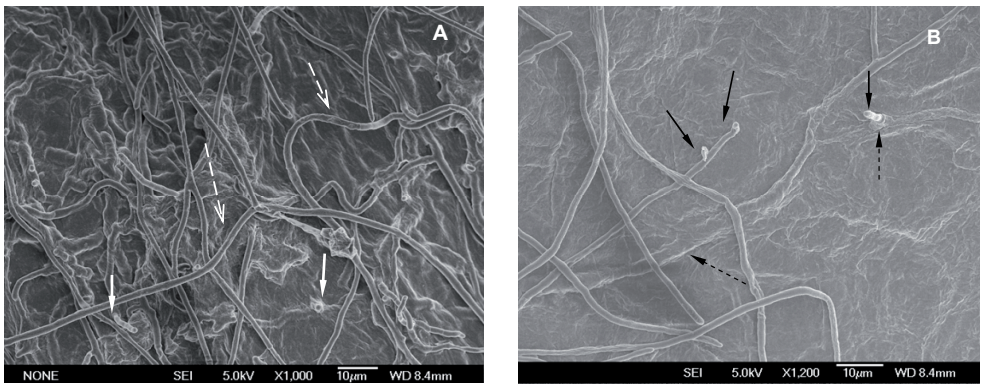

Figure 6.8. Visualisation of *T. rubrum* after a PDT with 200 µM Sylsens B displaying an incomplete fungal inactivation. Notice in A (white closed arrows) the points were fungal re-growth starts on certain parts of the treated fungus, visible here at the background. In front of this image normally growing fungal hyphae can be detected as well (white dashed arrows). In B the black dashed arrows point to a wrinkled, dented mycelium part, inactivated by PDT, while the closed black arrows point to the places where fungal re-growth started.

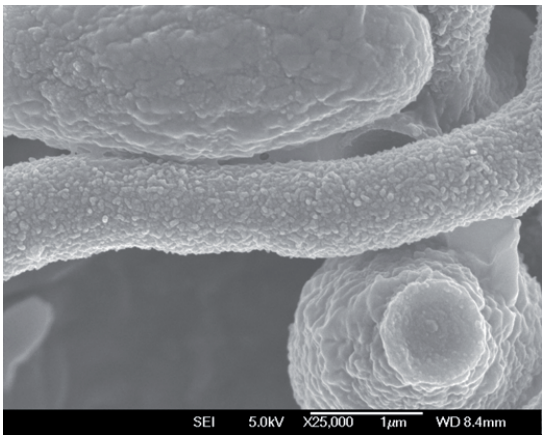

Figure 6.9. A SEM observation of *T. rubrum* fungal hyphae between two microconidia that are not yet germinated.

DISCUSSION

Dermatophytes exhibit various morphologies depending on their growth stage and environmental conditions (36). According to Vasquez et al., the surface of micro- and macroconidia of the genus *Trichophyton*, when cultivated under *in vitro* conditions, appears to be smooth. Bibel et al. described the ruffled surface of *Trichophyton mentagrophytes* when cultivated *in vitro* on Sabouraud dextrose agar (37), while Osumi described a granular surface cell wall on *T. rubrum* (38). In our studies, the surface of both the germinated microconidia and the hyphae had a rough, granular and fiber-mesh like appearance. Shortly after the microconidia adherence to the SC (but before germ tube development) the spore wall appeared to be more globular with a lack of fiber-like material and coated with a clear membrane (see Fig. 6.9 to illustrate this difference). This different structure could be necessary to enable attachment to the SC (39). The fungal wall structure on hyphal tips also differed from the rest of the hyphae i.e. a densely packed globular structure. We noticed this typical structure of the tips only at 72 hours after spore inoculation. This might indicate an increased metabolic activity at the tip of the hyphae.

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

In general, in all the different fungal growth stages, the observed damage due to the different treatments occurred shortly after the treatment. This is, in the case of PDT, to be expected because of the short lifetime of the reactive $^1O_2$. Although similar damage was observed after all the different treatments, only after PDT was the extent and severity such that it resulted in fungal death. The main consequences of an effective PDT were indented and deformed fungal elements, bulge formation, rupture of the fungal hyphae and, eventually, the development of areas displaying leakage of internal cell material. This sequence of events must have been caused at first by both the binding of the cationic porphyrin Sylsens B in the dark (during the incubation) to the fungal wall, followed by the production of $^1O_2$ after light application. Indeed, from the results of the dark treatments a minor bulge and leakage formation was observed, giving rise to some decrease in wall stability. Binding of Sylsens B in the dark to the fungal wall could decrease the binding possibilities for other cationic molecules involved in wall stability and also transport across the cell. This could also explain the growth delay after dark treatment with Sylsens B and the dark toxicity sporadically observed in earlier studies when Sylsens B was used at 200 µM concentration (16). Treatment with 108 J/cm^2 of red light alone (i.e. no photosensitizer) however, did not cause a growth delay in our previous experiments (16). This indicates that the observed injury induced by the red light had only a minor influence on fungal life.

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

We also looked more closely at the effect on fungal structure of only partly effective PDT (Fig 6.8). The SEM study confirmed our previous observation, that the unsuccessful PDT (80 to 90 % fungal kill) caused an arrest of the fungal growth (23). However, the fungal body was able to recover from certain surviving regions of the hyphae. As illustrated in Fig. 6.8 re-growth was observed on hyphal tips and in other hyphal areas. These observations can be of practical importance to PDT of *T. rubrum* applied at the time of full mycelium growth. The metabolically active fragments of hyphae are probably more resistant to PDT, either because of their better anti-oxidative defense due to the altered binding capacity for Sylsens B, or because of an increased keratinase enzyme production during this growth stage. Recently, we have shown that the inhibition of keratinase activity increased PDT efficacy when applied on *T. rubrum* mycelium at 72 hours after spore inoculation (23). In the present study, we also demonstrate a

difference in wall structure of the hyphal tips in full mycelium at 72 hours after spore inoculation when compared to the rest of the fungal hyphae and the tips during earlier stages. A consequence of these structural differences may be a decreased binding capacity of Sylsens B to these hyphal tips. This may result in lower local PDT effect and, consequently, the observed fungal re-growth. A similarly low binding capacity for Sylsens B might be present in the hyphae branching areas. Our SEM study indicated a difference in wall structure in branching areas (more globular and dense) compared to other hyphae regions. In other types of fungi, such differences were also observed; these morphologic differences were comparable with those found for the hyphal tips (40,41). These observations in fungal wall structure differences may be of importance for the treatment strategy. We hypothesize that better fungicidal effect could be achieved by application of a second treatment within 24 to 48 hours, a time interval in which hyphal tip morphology could not yet change into more resistant globular structures. However, further study is warranted to confirm this hypothesis.
Finally, it must be mentioned that although the photosensitizer concentrations used were known (from previous work) to be lethal and this was checked again in the present study at 8 days after spore inoculation, the present research has no correlation to clinical data.

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Chapter VII

DEVELOPMENT OF A TEST SYSTEM FOR MUTAGENICITY OF PHOTOSENSITIZERS USING *DROSOPHILA MELANOGASTER*

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Two *Trichophyton rubrum* mircroconidia, destroyed by photodynamic treatment with Sylsens B and red light shortly after germination

ABSTRACT

In the past few years, there has been an increase in the application of photosensitizers for medical purposes. A good standardized test system for the evaluation of the mutagenic potentials of photosensitizers is therefore an indispensable device. In the standard Ames test, white light itself was proven to be mutagenic and the result influenced by the light source. Lack of a reliable positive control is another problem in many genotoxicity test systems used for the evaluation of mutagenicity of photosensitizers. 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, 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 used to detect photogenotoxicity caused by short-lived photoproducts or by stable photoproducts or both. We demonstrated the Photo-SMART to be a good standardized test system for the evaluation of mutagenic potentials of the photosensitizer Sylsens B. We demonstrated that Sylsens B was mutagenic using the Photo-SMART. For hematoporphyrin, the results of the Photo-SMART indicate the absence of mutagenicity.

INTRODUCTION

In the past few years, there has been a great improvement in the development of new photosensitizers and their application for medical purposes (1,2). At present, it is therefore necessary to evaluate the different phototoxic effects of photosensitizers in preclinical studies. With respect to this subject, the reported DNA damage and inhibition of DNA repair functions by photooxidative reactions is an interesting aspect (3). Mutagenic potentials of photosensitizers used in PDT are an important issue to which much attention has been paid (4-6). The need for a good and standardized test system for photomutagenicity is an essential qualification.

Till date, many different test systems for the detection of photochemical genotoxicity have been reported (7), but most of them have certain limitations. In the well-known and internationally accepted Ames test, although correctly adjusted to use light sensitization (8,9), white light was proven to induce mutagenicity as well (10). Another problem is the lack of a reliable positive control for the photomutagenicity test system.

An important qualification of the photomutagenicity test system is the possibility to detect within one system photogenotoxicity caused by either the production of short-lived products, such as reactive oxygen species (ROS), or the production of stable photoproducts. The aim of the present study was to introduce a test system that in the first place complies with these terms, in which white light itself does not display mutagenicity and that offers a standard positive control. We investigated the possibility of methylene blue (MB, Table 7.1), known to induce photomutagenicity (7,11) to act as a reliable positive control in our test system. Furthermore, the test system we developed, based on the validated somatic mutation and recombination test (SMART) (12,13) and referred to as Photo-SMART, uses a whole organism, *Drosophila melanogaster*. More than 180 chemicals were tested in the SMART (13), and the results were in accordance with the data given by other tests. The SMART is an important test in *D. melanogaster* for the identification and characterization of potential genotoxic compounds and scores for loss of heterozygosity (LOH) (14). LOH may occur due to different types of recombination, deletions, point mutations, loss of chromosomes or nondisjunction. In flies heterozygote for the recessive eye color marker *white*, new somatic mutations of the wild-type *white* gene, formed early in the larval development, become visible as white spots in the otherwise normally red colored eyes. Increased sensitivity of the test was obtained by introduction of the

tumor suppression gene *warts* (14,15) and evaluated with the chemical mutagen methylmethanosulfonate and X-rays (14). Loss of the wts^+ function in wts/wts^+ cells results in overproliferation and apical hypertrophy of epithelial cells, producing a wart-like phenotype on all external parts of the flies including the eyes.

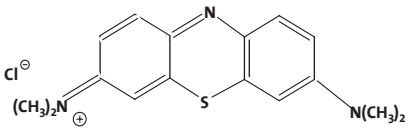
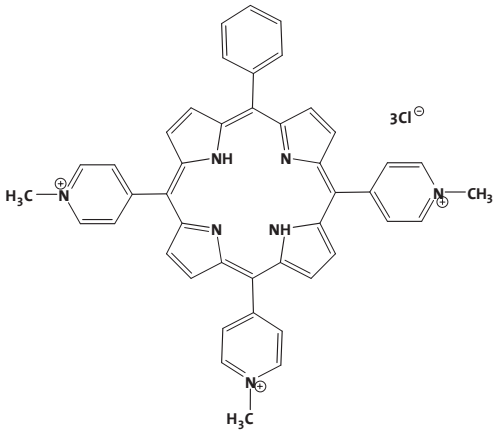
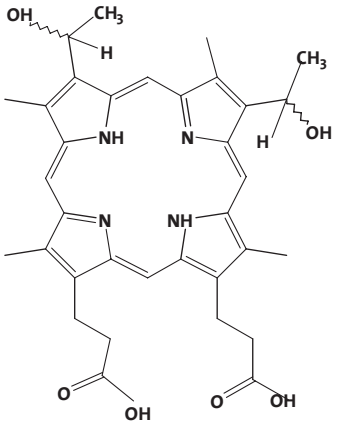
Chemical Structure	*Selected name*	*Abbreviation*
	Methylene Blue	MB
	5,10,15-tris(4-methylpyridinium)-20-phenyl-[21H,23H]-porphine trichloride	Sylsens B
	Hematoporphyrin	HP

Table 7.1. Formulae of the porphyrin photosensitizers, Sylsens B and HP and the phenotiazine MB

Use of a whole organism is an important additional amenity of the test. This is especially true because it was recently demonstrated that *D. melanogaster* could be used to assess photodynamic activity of many different photosensitizers as well (G. M. T. Smijs, unpublished). It is important to realize that the Photo-SMART is meant to detect certain biological effects caused by photosensitizers under comparable circumstances. To evaluate the Photo-SMART, the mutagenetic potentials of the well-known photosensitizers hematoporphyrin (HP) and Sylsens B were investigated. The absorption maximum for HP is situated at 392 nm, for Sylsens B at 424 nm and for MB at 661 nm. For formulae and related names, see Table 5.1. In all cases, presence or absence of mutagenicity was determined using experimental conditions such that between 40% and 60% light-dependent kill occurred.

MATERIALS AND METHODS

Strains and crosses

The following *D. melanogaster* strains were used: a strain carrying the X-linked eye color marker white (*w*) and a strain carrying on the third chromosome $wts^{MT4\text{-}1}$, a lethal *warts* allele, balanced over TM3, characterized by multiple inversions and marked by the dominant mutation *stubble* (*Sb*). The warts allele $wts^{MT4\text{-}1}$ was induced with the alkylating agent ethylmethanesulfonate in a chromosome carrying the eye color markers starlet and pink, the bristle marker inturned and the wing morphology marker radius incomplete (14,16).

The following cross was made: *wts*/TM3 males with *w* females.

Chemicals

HP was from Porphyrin Products Inc. (Logan, UT), Sylsens B was synthesized and kindly provided by ARC Laboratories BV at the Department of Bio-Organic Photochemistry, Leiden University, The Netherlands (purity, checked with nuclear magnetic resonance, was more than 99.5%) and MB was purchased from J.T. Baker (Deventer, The Netherlands). Tween-80 was obtained from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands), whereas all other chemicals were from J.T. Baker.

The following solvent was used for the photosensitizers: a sodium-potassium phosphate buffer, pH 7.0 (2.79 g Na_2HPO_4 and 2.27 g KH_2PO_4/L distilled water). Stock solutions of the photosensitizers HP (20 mM), Sylsens B (20 mM) and MB (5 mM) were stored at 4°C for no longer than 1 week.

Light source

Illuminations were performed with a lamp from MASSIVE (no. 74900/21), 1 x maximum 500 W, 230 V, R7s, IP 44. To avoid heating of the samples to be illuminated, the white light produced by the lamp was passed through water layer first before reaching the samples. Light intensity was measured with IL1400A photometer equipped with a SEL033/F/U detector (International Light, Newburyport, MA). The amount of UVA and UVB in the white light produced by the lamp was measured with an UVX radiometer; UVP, Upland, CA). The amount of UVA appeared to be less than 0.1%, and the amount of UVB appeared to be 0.007%.

Photo-SMART

The method used and referred to as Photo-SMART is a modification of the SMART, which is a combination of the *w/w*$^{+}$ eye test (10) and the *wts/wts*$^{+}$ tumour suppressor system (15).

w females (40) flies were mated with *wts*/TM3 males (35) flies and allowed to lay eggs in bottles on food supplemented with the photosensitizer dissolved in solvent before mixing it to the standard food (2 mL/50 mL food). After 24 h, the parent flies were discarded, and the eggs were allowed to develop for another 75 h. The larvae were then isolated with a 20% sucrose solution, washed twice with a 0.7% NaCl solution (using a 30 µM Millipore filter) and illuminated in a total volume of 2 mL of 10% sucrose solution in 6 cm diameter culture dishes (Greiner, Alphen aan de Rijn, The Netherlands). Reduction of the light through the plastic lid was evaluated and found to be negligible. Light fluency and concentration range were always such that a light-dependent kill of 40–60% occurred. These conditions have to be established in preliminary experiments using the same experimental setup and parental cross.

After illumination or dark period, treated larvae were transferred to bottles containing normal food supply and left to develop at 25°C. After 10 days, the number of flies present in the bottles was counted and males were removed and females were collected (genotypes, *w/w*$^{+}$ and *w/w*$^{+}$*; wts/wts*$^{+}$) for scoring of mutagenicity. The scoring of etherized flies was carried out in a liquid consisting of 90 parts of ethanol, one part Tween-80 and nine parts water. Inspection of the eyes for the presence of white spots and warts tumors (Fig. 7.1) was performed using a Nikon stereomicroscope at a magnification of 50–75x. Large white spots are seen as white parts in the red eyes and small white spots as dark ommatidia in a red eye. Similarly, small warts tumours are seen as dark ommatidia. Spots separated from each other by at least four

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nonmutated ommatidia were counted as independent events, and the smallest size of white clone expected to be counted accurately is 2 (13,17). The white spots and warts tumours were scored together. An increase in white and warts clone frequency was only accepted as a positive response if it was significantly higher than that of both the concurrent and pooled experimental controls.

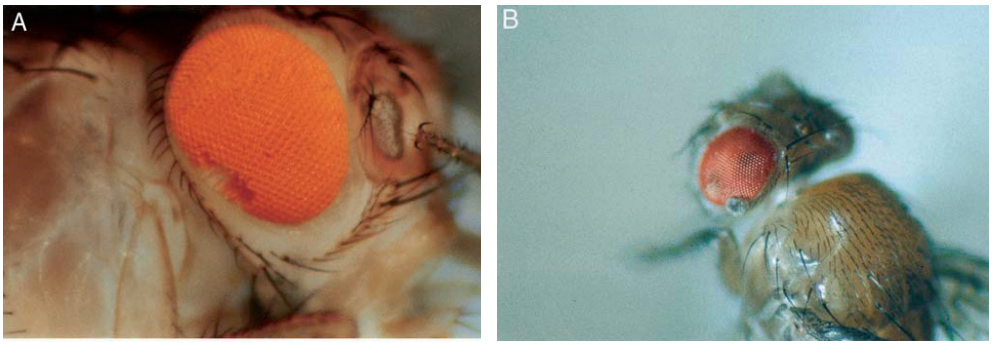

Figure 7.1. Drosophila eye (A) showing a large white spot in the lower left quadrant and an eye (B) showing a warts tumor.

Statistical test

The statistical significance of the differences between spot frequencies in the experiment (light induced) and control (dark) was calculated using the X^2 test for proportions as described by Frei and Würgler (18).

RESULTS

White light

For both genotypes scored in the Photo-SMART, *w/w⁺; wts/wts⁺* and the *w/w⁺*, no mutagenicity was established for the white light itself. For the different light doses used, namely 180, 360 and 1080 kJ/m², the scored spot percentage was not significantly [X^2 method, (18)] higher than that scored for the corresponding dark controls (Tables 7.2–7.4).

MB concentration (mM)	*0*	*0.01*	*0.025*	*0.05*	*0.1*	*0.2*
A: ***w/w⁺;wts/wts⁺***						
N_t	400	290	320	420	514	620
n_t	50	75	99	166	245	372
N_c	400	600	600	600	600	600
n_c	66	84	78	92	80	96
χ^2	1.91	14,7	33,9	60,4	110,6	152,7
B: ***w/w⁺***						
N_t	400	222	276	410	480	446
n_t	33	45	65	115	154	163
N_c	400	400	400	260	400	400
n_c	40	44	44	33	36	40
χ^2	0.49	7,9	18,4	16,3	52,78	60,8

Table 7.2. Test of statistical significance of the data for MB using the X^2 test as described by Frei and Würgler (18). The result for the *w/w⁺; wts/wts⁺* system is given in A (corresponding the filled and open squares in Fig. 7.3) and for the *w/w⁺* in B (corresponding filled and open diamonds in Fig. 7.3)[a]
[a] N_t = number of eyes scored in the test, i.e. the number of eyes of the flies that were submitted to light treatment (1080 kJ/m²). n_t = number of spots scored in the test, i.e. the number of spots scored in the eyes of the flies that were submitted to light treatment (1080 kJ/m²). N_c = number of eyes scored in the control, i.e. the number of eyes of the flies that were kept in the dark. n_c = number of spots scored in the control, i.e. the number of spots scored in the flies that were kept in the dark. X^2 (α = 0.05; ν = 1) = 2.706.

HP concentration (mM)	*0*	*0.1*	*0.3*	*0.6*	*0.8*
A: ***w/w⁺;wts/wts⁺***					
N_t	400	180	300	200	100
n_t	40	28	63	26	20
N_c	400	200	200	200	200
n_c	44	20	22	18	22
χ^2	0.11	0.76	6,48	1,11	3,24
B: ***w/w⁺***					
N_t	400	144	300	200	190
n_t	28	8	15	10	24
N_c	400	200	300	200	200
n_c	28	16	14	10	16
χ^2	0.02	0,409	0	0	1,6

Table 7.3. Test of statistical significance of the data for HP using the X^2 test as described by Frei and Würgler (18). The result for the *w/w⁺; wts/wts⁺* system is given in A (corresponding filled and open squares in Fig. 7.5) and for *w/w⁺* in B (corresponding filled and open diamonds in Fig. 7.5)[a]
[a] N_t = number of eyes scored in the test, i.e. the number of eyes of the flies that were submitted to light treatment (360 kJ/m²). n_t = number of spots scored in the test, i.e. the number of spots scored in the eyes of the flies that were submitted to light treatment (360 kJ/m²). N_c = number of eyes scored in the control, i.e. the number of eyes of the flies that were kept in the dark. n_c = number of spots scored in the control, i.e. the number of spots scored in the flies that were kept in the dark. X^2 (α = 0.05; ν = 1) = 2.706.

Sylsens B Concentration (mM)	*0*	*0.1*	*0.4*	*0.6*	*0.8*
A: ***w/w⁺ ;wts/wts⁺***					
N_t	400	400	400	400	162
n_t	54	111	133	155	58
N_c	400	300	300	300	300
n_c	48	48	44	54	53
χ^2	0.25	10	22,6	24,0	13,6
B: ***w/w⁺***					
N_t	400	400	380	400	210
n_t	32	80	104	115	71
N_c	400	200	180	200	160
n_c	28	22	28	34	26
χ^2	0.15	5,8	6,7	6,9	10,0

Table 7.4. Test of statistical significance of the data for Sylsens B using the X^2 test as described by Frei and Würgler (18). The result for the *w/w⁺; wts/wts⁺* system is given in A (corresponding filled and open squares in Fig.7. 6) and for the *w/w⁺* in B (corresponding filled and open diamonds lines in Fig. 7.6)[a]
[a] N_t = number of eyes scored in the test, i.e. the number of eyes of the flies that were submitted to light treatment (180 kJ/m²). n_t = number of spots scored in the test, i.e. the number of spots scored in the eyes of the flies that were submitted to light treatment (180 kJ/m²). N_c = number of eyes scored in the control, i.e. the number of eyes of the flies that were kept in the dark. n_c = number of spots scored in the control, i.e. the number of spots scored in the flies that were kept in the dark. X^2 (α = 0.05; ν = 1) = 2.706.

Methylene blue

The experimental conditions used in the photomutagenicity test, the Photo-SMART, were determined in a number of preliminary experiments. These experiments comprise light dose and concentration variations using the experimental setup as described for the Photo-SMART. The results of these experiments yielded a number of combinations of light dose and MB concentration, each resulting in a light-dependent kill of about 50%. Based on the final results, as shown in Fig. 7.2, and the practical circumstances, a light dose of 1080 kJ/m² was chosen for MB. At this particular light dose and final MB concentration of 0.2 mM, a light-dependent kill of 50% was observed. The practical circumstances referred to comprise the solubility of the photosensitizer and the heat production of the lamp. When using higher MB concentrations, it is the dark toxicity that becomes increasingly important.

In a total of three different experiments, we determined the mutagenicity of MB in the Photo-SMART as described using a light fluency of 1080 kJ/m² and a concentration range that varied up to 0.2 mM (Fig. 7.3). Before scoring of the mutagenicity, the survival of *D. melanogaster* at each different MB concentration was determined. The

results were in concordance with the results obtained in the preliminary toxicity experiments. Up to 0.025 mM MB, we found a light-dependent survival of 100%, 0.050 mM yielded 90% survival, 0.1 mM 65% and 0.2 mM 50%. The dark toxicity is negligible for MB concentrations up to 0.1 mM and ranges between 20% and 25% for 0.2 mM MB.

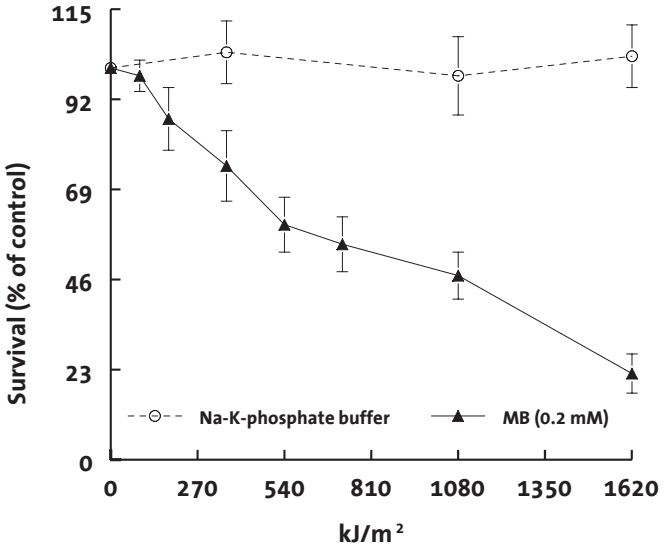

Figure 7.2. Survival curves for *Drosophila melanogaster* exposed to increasing levels of white light at a constant concentration of MB of 0.2 mM, using the parental cross *wts*/TM3 males with *w* females. White light intensity variations were performed at a fixed MB concentration of 0.2 mM. In the control experiment, the solvent, phosphate buffer of pH 7.0, was used instead of MB. The number of flies in the control, no light administration, was 184 for MB and 250 for the solvent. The values given here are the mean of three experiments and the standard deviation and represent the number of flies counted 10 days after the start of the experiment.

As can be seen from Fig. 7.3, there is definitely a concentration dependent increase in spot percentage in both genotypes that were scored, the *w/w*$^+$ and *w/w*$^+$*; wts/wts*$^+$ system.

Significance of the results obtained for the light-induced mutagenicity and the dark control values was determined according the X^2 method as described by Frei and Würgler (18). A summary is given in Table 7.2 for both the *w/w*$^+$ and *w/w*$^+$*; wts/wts*$^+$ system (A) and the *w/w*$^+$ (B). In both cases, significance (for α = 0.05; ν = 1, the value for X^2 is 2.706) is evident for all the concentrations submitted to the test.

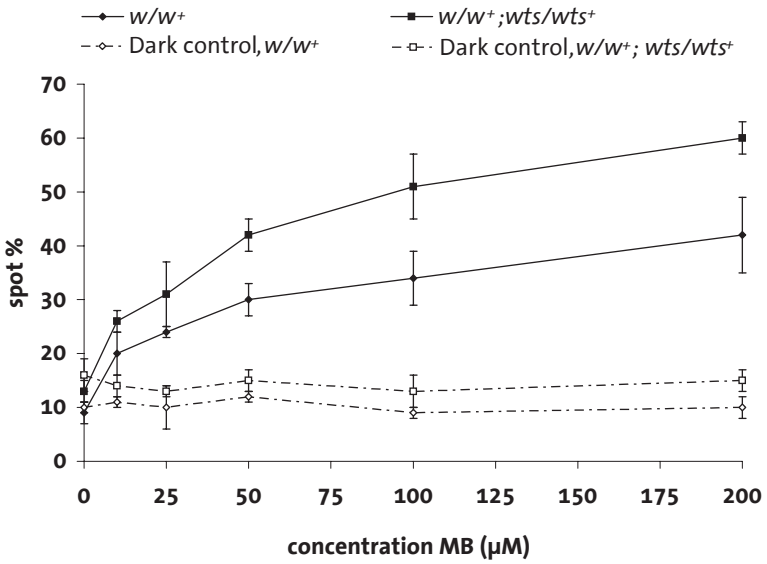

Figure 7.3. Light-induced increase in the percentage of white spots and warts tumors for MB according to the Photo-SMART.
Light conditions: white light, 1080 kJ/m² (UVA, 0.1%, UVB, 0.007%). The values given are the mean of three different experiments and the standard deviation and represent the percentage of white spots (*w/w⁺*), or white spots and warts tumors (*w/w⁺; wts/wt*), of the number of counted eyes. The number of eyes scored in one experiment varied from 100 to 200 for a given concentration and genotype.

Other photosensitizers

To evaluate the test system for photomutagenicity further, the two porphyrin photosensitizers, Sylsens B and HP, were submitted to the system to investigate their mutagenetic potentials.

From the preliminary experiments, different combinations of light dose and photosensitizer concentration were obtained, all resulting in a 50% kill. Practical circumstances, solubility properties of the photosensitizers and the presence of dark toxicity with higher concentrations, will eventually determine the combination to be used in the Photo-SMART. Figure 7.4 gives the final result of the preliminary experiments concerning the light conditions to be used in the Photo-SMART. As can be seen from this figure, in both cases, a photosensitizer concentration of 0.7 mM was used, and a variable light dose of up to 2160 kJ/m² for HP and 1620 kJ/m² for Sylsens B was used. At this photosensitizer concentration, light conditions giving 50% light-dependent kill appeared to be 360 kJ/m² for HP and 180 kJ/m² for Sylsens B. For higher photosensitizer concentrations, solubility problems became important for both sensitizers, and especially for Sylsens B the dark toxicity.

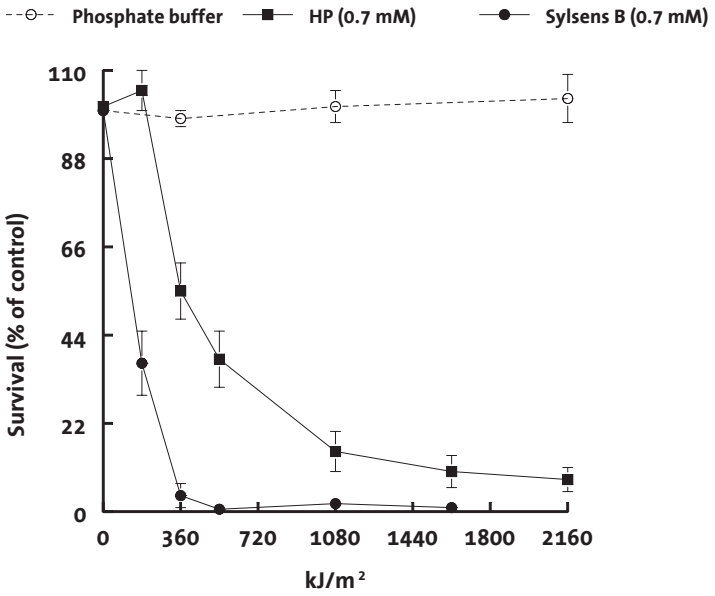

Figure 7.4. Survival of *Drosophila melanogaster* as a function of various doses of white light at a constant concentration of HP or Sylsens B of 0.7 mM, using the parental cross *wts*/TM3 males with *w* females. White light variations were performed at a fixed photosensitizer concentration of 0.7 mM. In the control experiment, the solvent, phosphate buffer of pH 7.0, was used instead of photosensitizer. The number of flies in the control, no light administration, was 225 for HP, 215 for Sylsens B and 250 for the solvent. The values given are the mean of three experiments and the standard deviation and represent the number of flies counted 10 days after the start of the experiment.

Two separate mutagenicity experiments were performed for both HP and Sylsens B varying the sensitizer concentration up to 0.8 mM. The mean result is given in Fig. 7.5 for HP and in Fig. 7.6 for Sylsens B. In the case of Sylsens B, an increase in spot percentage can be seen as a result of an increasing photosensitizer concentration. Significance was determined as described (17), and the results are summarized in Table 7.3 for HP and in Table 7.4 for Sylsens B.

Table 7.3 shows that for HP in the *w/w⁺; wts/wts⁺* system, only the concentrations 0.3 and 0.8 mM led to a significant spot enhancement in the presence of white light (for α = 0.05; ν = 1, the value for X^2 is 2.706). In the other cases (0.1 and 0.6 mM), no significant spot enhancement could be detected. For the *w/w⁺*, no significant increase in spot percentage could be detected for HP for the whole concentration range, when compared with the dark control values.

From Table 7.4, it may become clear that in the case of Sylsens B, there is definitely a significant difference in spot percentage for both the *w/w⁺; wts/wts⁺* system (A) and the *w/w⁺*, (B).

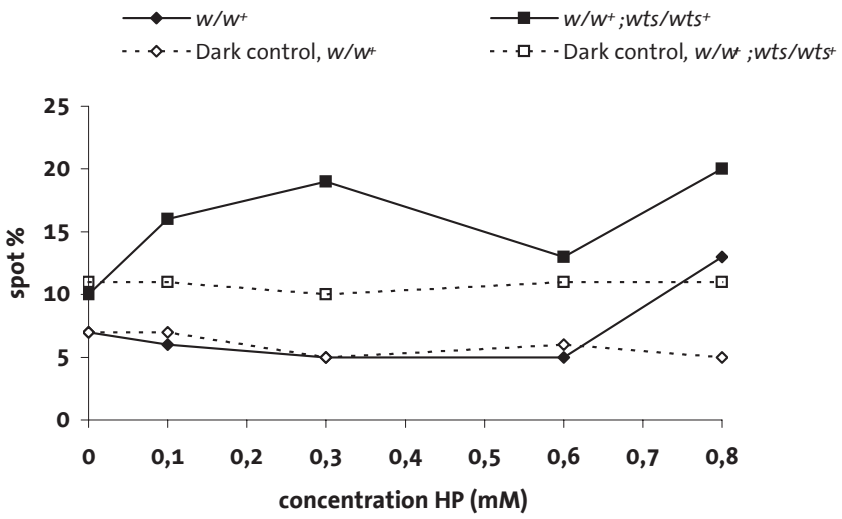

Figure 7.5. Percentage of white spots and warts tumors at a constant dose of white light and increasing concentrations of HP Light conditions: white light, 360 kJ/m² (UVA, 0.1%, UVB, 0.007%). The values given are the mean of two different experiments and represent the percentage of white spots (*w/w⁺*), or white spots and warts tumors (*w/w⁺; wts/wt*), of the number of counted eyes. The number of eyes scored in one experiment varied from 100 to 200 for a given concentration and genotype.

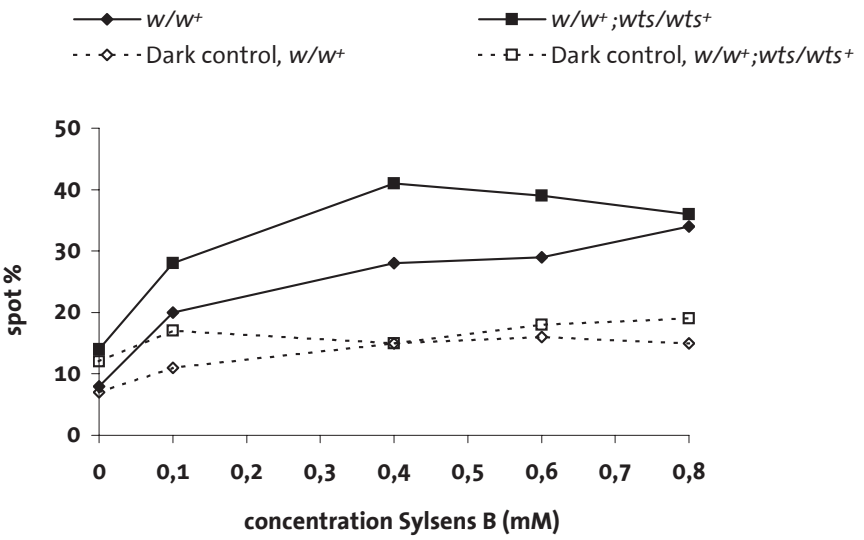

Figure 7.6. Percentage of white spots and warts tumors at a constant dose of white light and increasing concentrations of Sylsens B. Light conditions: white light, 180 kJ/m² (UVA, 0.1%, UVB, 0.007%). The values given are the mean of two different experiments and represent the percentage of white spots (*w/w⁺*), or white spots and warts tumors (*w/w⁺; wts/wt⁺*), of the number of counted eyes. The number of eyes scored in one experiment varied from 100 to 200 for a given concentration and genotype.

The determination of the light-dependent survival of *D. melanogaster*, before scoring of mutagenicity, yielded for HP 100% survival for 0.1 mM, 90% for 0.3 mM, 70% for 0.6 mM and 60% for 0.8 mM. For Sylsens B, the light-dependent survival values were 90% for 0.1 mM, 70% for 0.4 mM, and between 40% and 60% for the highest concentrations of 0.6 and 0.8 mM. Dark toxicity for HP was found to be negligible in the given concentration range. For Sylsens B, the dark toxicity is negligible up to a Sylsens B concentration of 0.4 mM, and between 10% and 15% for the highest concentrations of 0.6 and 0.8 mM.

DISCUSSION

Clearly, white light itself did not induce mutagenetic events, leading to an increase in spot percentage, in the presented Photo-SMART. The presented data therefore provide the evidence of a genotoxic effect from PDT mediated by MB in Drosophila eye tissue. As mentioned before, the amount of UVA and UVB produced by the light source used in the experiments was measured. The amounts of UVA and UVB were such that no mutagenic effects in Drosophila were to be expected [(19,20); M. J. M. Nivard, unpublished]. Moreover, the experimental setup is such that the sample spot is situated beneath glass. A glass filter containing water (5 cm thickness) is used to absorb the infrared light from the lamp.

It is important to realize that the presented test system is meant to provide a method for the evaluation of the mutagenic potentials of medical photosensitizers. This category of photosensitizers will comprise preferably no chemicals that possess substantial absorption peaks in the UV part of the spectrum because of the low penetration capacity of the UV light in tissue.

The results obtained for MB with the Photo-SMART, as presented in Fig. 7.3, show that there is definitely a concentration and light-dependent increase in spot percentage. An increase in spot percentage was shown to be significantly higher (Table 7.2) than the spot percentage obtained for the dark control (for every tested photosensitizer concentration).

The Photo-SMART as described here is, to our opinion, a valuable tool to evaluate mutagenetic potentials of photosensitizers. As mentioned in the Introduction, this test system provides reliable information about biological effects caused by photosensitizers. The test offers the possibility to compare the positive biological

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effects, the light-induced toxicity, with the negative effects, the mutagenicity. Because the light treatment in the Photo-SMART is completely included within the test system, this system offers the possibility to detect not only mutagenic potential of stable photoproducts but also the mutagenic potential of short-lived photoproducts, such as ROS. Using a whole organism in this test may of course have great advantages compared with the customary genotoxicity tests. It is commonly accepted now, in considering mutagenicity testing, to regard a negative finding in an *in vivo* procedure as the definitive indicator of the lack of genotoxic potential, whatever the effects seen in *in vitro* studies.

An additional advantage of the Photo-SMART is the fact that *D. melanogaster* was recently proven to be susceptible to PDT (G. M. T. Smijs, unpublished) and thus can act as a valuable tool in preclinical PDT studies. Measurements of photodynamic efficacy, uptake and clearance and the mutagenic potential of chemicals can thus be performed using the same test organism. This offers the possibility to correlate pharmacokinetic studies to photodynamic and mutagenic studies.

The results obtained for HP and Sylsens B in the Photo-SMART led to the conclusion that there is definitely an indication for mutagenicity for Sylsens B under the given conditions (Table 7.4, Fig. 7.6). For HP, however, the presented data (Table 7.3, Fig. 7.5) give no elucidation concerning the presence of mutagenicity. Additional experiments, leading to a larger total amount of scored eyes (N_t and N_c), will be necessary to present the necessary explanation. Another possibility may comprise experiments using a lower HP concentration in combination with a higher light dose. In that case, however, photobleaching characteristics of HP might interfere with the thus obtained results (21).

Considering all the data obtained here using the Photo-SMART, it is obvious that the introduction of the warts tumor makes the test system more sensitive, as was reported before (14,15). Important in this aspect is the fact that the tumour suppressor gene warts (*wts*) is involved in cell cycle regulation, and homologues of the *wts* gene are also found in lower organisms (22). The possibility of isolating the easily detectable *wts* tumors, produced in the Photo-SMART, and the possibility to submit them to further study, offers a valuable advantage of this test.

We conclude that because white light was found to be not mutagenic and the fact that a whole organism is used as test organism, the presented Photo-SMART may be seen as a valuable addition to the current range of photogenotoxicity test systems.

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Chapter VIII

INVESTIGATION OF THE BEHAVIOUR OF THE CATIONIC PHOTOSENSITIZER 5,10,15-TRIS (4-METHYLPYRIDINIUM)-20-PHENYL-[21*H*,23*H*]-PORPHINE TRICHLORIDE AFTER ITS APPLICATION ON HEALTHY SKIN AND STRATUM CORNEUM INFECTED WITH THE DERMATOPHYTE *TRICHOPHYTON RUBRUM*

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Submitted

Trichophyton rubrum hyphae after photodynamic treatment with a lethal Sylsens B concentration, showing a complete loss of the fibre-mesh wall material

ABSTRACT

Dermatophytes cause superficial infections of keratinized tissues. Recently, we have demonstrated that the most frequently occurring dermatophyte, *Trichophyton rubrum*, could be destroyed with photodynamic treatment (PDT) with a 5,10,15-tris (4-methylpyridinium)-20-phenyl-[21*H*,23*H*]-porphine trichloride (Sylsens B) formulation with low ion content and pH 5.2. For a save application of Sylsens B in clinical PDT of dermatophytoses it is neither necessary nor desirable that Sylsens B penetrates the skin. To investigate whether this effective Sylsens B formulation could guarantee low Sylsens B penetration in healthy skin and skin infected with *T. rubrum*.

Sylsens B skin penetration studies were performed with dermatomed skin, human stratum corneum (SC), disrupted human SC by *T. rubrum* growth and human SC pre-treated with a detergent. The effective Sylsens B formulation (pH 5.2) was compared to a formulation in PBS (pH 7.4). Visualisation in dermatomed skin was performed with confocal scanning laser microscopy (CSLM).

There was no Sylsens B penetration in healthy skin at pH 7.4 or 5.2. Disruption of SC by preceding fungal growth caused Sylsens B to penetrate at pH 7.4, but not in case of our PDT formulation with pH 5.2, while chemically damaged SC caused Sylsens B penetration also at pH 5.2. CSLM investigations confirmed that in dermatomed skin Sylsens B did not reach viable epidermis and dermis.

The presence of *T. rubrum* on SC prevented Sylsens B to penetrate when using the effective PDT formulation (low ion content and pH 5.2). Therefore, this formulation may be safe for a future clinical PDT of dermatophytoses caused by *T. rubrum*.

INTRODUCTION

Dermatophytes are fungi that cause infections of superficial keratinized tissues (1,2). These infectious agents rarely invade deeper layers of the skin. The most commonly isolated dermatophyte with worldwide distribution is *Trichophyton rubrum* (3,4).

The present treatment strategies (topical and/or oral drug application or a combination of both) are not always curative (5,6). One of the reasons is that the current treatments inhibit mainly the metabolic active fungus (1,7-9), and leave the spores unaffected. 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 (10).

Recently, we have demonstrated that a single photodynamic treatment (PDT) with the photosensitizer 5,10,15-tris(4-methylpyridinium)-20-phenyl-[21*H*,23*H*]-porphine trichloride (Sylsens B, Fig 8.1) applied to *T. rubrum* grown on human stratum corneum (SC) resulted in complete fungal kill (fungicidal effect) (11,12).

Photodynamic treatment refers to the use of light-activated agents called photosensitizers (13). Upon irradiation with light of an appropriate wavelength, photosensitizers can initiate a photochemical reaction resulting in the production of reactive oxygen species. This sequence of these events is known as photodynamic effect and it can cause the elimination of pathogens (14). The use of PDT for fungal infections is a new and promising approach (12,15). Effective PDT requires a selective binding of the photosensitizer to the target organism (14,16). In case of *T. rubrum,* our previous results showed that the fungicidal PDT effect was indeed achieved after the selective binding of the positively charged porphyrin Sylsens B to the negatively charged outer wall of fungal microconidia or hyphae. This binding could be accomplished with a Sylsens B formulation when low ion strength and a pH of 5.2 were used (12).

For a topical application of Sylsens B in patients with dermatophytosis it is neither necessary nor desirable that Sylsens B penetrates through the SC into the viable epidermis and dermis. A formulation that does not cause skin penetration of Sylsens B in neither healthy nor dermatophytosis infected skin is therefore to be preferred in future clinical experiments.

We therefore investigated the penetration behaviour of Sylsens B in healthy dermatomed skin, normal human SC, human SC infected with *T. rubrum* microconidia, and human SC pre-treated with a detergent. Reproducible fungal growth on SC was achieved by utilizing two fungal growth stages that corresponded to 3 and 5 days after the spore inoculation. The permeation studies were performed with Sylsens B in

two formulations: i) the previously successfully tested formulation containing a low molarity buffer of pH 5.2 (12) and ii) Sylsens B in phosphate buffered saline (PBS), at pH 7.4. Since it has been reported that sodium lauryl sulphate (SLS) reduces the barrier function of the SC (17), we also examined (as a positive control) the penetration of Sylsens B (at pH 5.2) through SC pre-treated with SLS.
In order to investigate whether Sylsens B could be visualized in SC and the viable skin, the dermatomed skin was cross-sectioned after the diffusion studies and visualized using confocal scanning laser microscopy (CSLM).

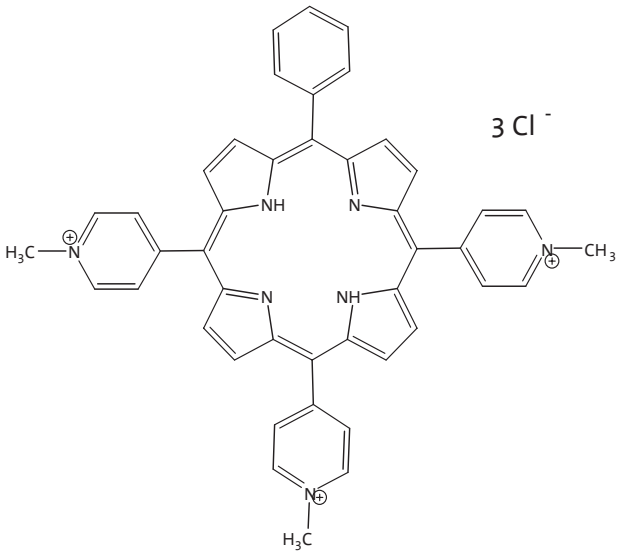

Figure 8.1. Chemical structure of the porphyrin photosensitizer 5,10,15-tris(4-methylpyridinium)-20-phenyl-[21*H*,23*H*]-porphine trichloride (Sylsens B).

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.
Sylsens B (mol wt: 769.16 g/mol) was synthesized by Buchem Holding BV (Lieren, The Netherlands). Its purity, determined with NMR was more than 99 %. Trypsine was

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obtained from Sigma (Zwijndrecht, The Netherlands), while all other chemicals were purchased from J.T.Baker (Deventer, The Netherlands).
Solutions of Sylsens B were made in either PBS (pH 7.4) or in a 5 mM citric acid/sodium citrate buffer (pH 5.2) and stored at 4°C for no longer than one week.

Preparation microconidia suspension

The protocol for obtaining a suspension of microconidia produced by *T. rubrum* grown on Sabouraud Dextrose Agar was based on a method described previously (11,18). The obtained microconidia suspensions (10– 40 x 10^6 colony forming units (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 human dermatomed skin and SC

Abdominal or breast 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 200 to 250 µm using a Padgett Electro Dermatome Model B (Kansas City, USA). For the preparation of the SC 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 hour at 37°C, the SC was removed manually. The obtained SC was dried in the air for 24 hours and kept under nitrogen over silicagel for no longer than 3 months.
For the penetration studies, circular sheets of 18 mm in diameter of dermatomed skin or SC were used. The SC was used either directly, after 24 hours pre-treatment with 1% SLS (followed by 5 washing steps to remove the SLS) or after infection with *T. rubrum*. For the latter purpose, the SC was placed in the central part of a polycarbonate membrane filter, 25 mm in diameter and a pore size of 2 µm (Omnilabo, Breda, The Netherlands) on a 3 cm culture dish (Greiner, Alphen aan den Rijn, The Netherlands) filled with 5 mL of Malt Extract Agar. A microconidia suspension was diluted to 175 cfu/ml and 20 µL inoculated in the centre of the SC. After the inoculation, the dish was placed in an incubator at 28°C for 3 or 5 days and the required number of fungal colonies checked microscopically prior to the penetration experiment. In this way, reproducible skin barrier damage could be obtained due to growth of 3 to 4 fungal colonies at 3 and 5 days after spore inoculation.

Penetration studies

In vitro penetration studies were performed using Teflon diffusion cells (see Fig. 8.2), designed and produced by the Department of Fine Mechanics of the University of Leiden. The diffusion area of the cells was 0.78 cm^2 and the volume of the acceptor chamber 0.98 cm^3.

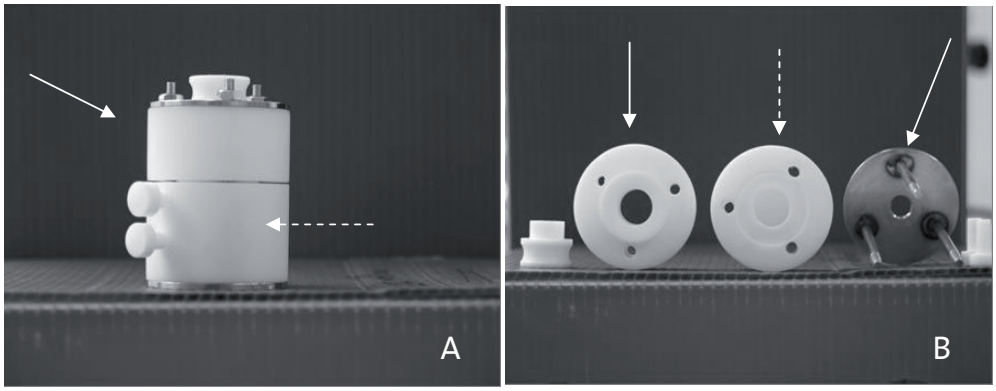

Figure 8.2. Photograph of the Teflon diffusion cell used for the penetration studies. The cell consists of an upper donor chamber (arrow in A and white left arrow in B) and a lower acceptor chamber (dashed arrow in A and B). The skin or SC is placed in the centre of the acceptor chamber that is subsequently filled with acceptor liquid. After filling the acceptor chamber and removal of air bubbles the chamber is closed. The donor chamber is mounted to the acceptor chamber, filled with donor liquid and finally the cell assembled with the screwing system as shown by the right arrow in B.

A circular piece of SC or dermatomed skin with a thickness of approximately 200 to 250 µm and a diameter of 18 mm was placed in the centre of the acceptor chamber. The acceptor chamber was filled either with PBS (pH 7.4) supplemented with 5 % ethanol or 5 mM citric acid/sodium citrate buffer (pH 5.2) with 5 % ethanol and the donor chamber with 300 µL Sylsens B solution of 80 and 160 µM (respectively 62 and 123 µg/ml). Controls contained a donor chamber filled with PBS (pH 7.4) or 5 mM citric acid citric acid/sodium citrate buffer (pH 5.2). The cells were placed for 24 hours (under occlusive conditions) in a water bath with a temperature of 32°C. Then before disassembling the cell the SC or dermatomed skin was washed with buffer and the acceptor volume was collected (syringe) and weighed. The concentration of Sylsens B in the acceptor phase was determined by measuring the fluorescence at 657 nm upon excitation at 424 nm (Perkin Elmer LS 50B luminescence spectrometer, Perkin Elmer Nederland BV). For every pH, a calibration curve was made to determine Sylsens B concentration in the acceptor phase. Each experiment contained 3 cells for each test

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condition and experiments were repeated at least 6 times using skin from different donors.

Confocal scanning laser microscopy

After the penetration experiments with dermatomed skin, the skin was cross-sectioned using a cutting device based on the device as described earlier (19) (illustrated in Fig. 8.3A). In addition to the device as described we included perplex holders to support the silicon sheets. The cross-sectioned skin was mounted in a sample holder positioning the cutting surface against the cover glass (Fig. 8.3B). The CSLM was carried out using a BioRad Radiance 2100 MP (BioRad, Hertfordshire UK) equipped with an Argon/HeNe laser. Samples were examined approximately 20 µm below the cutting surface using a 514 nm laser line with a Flaur 40x 1.30 oil immersion objective. Confocal images were collected (at least 10 for every condition) and digitized using Zeiss Lasersharp 2000 and the image-Pro 3Ds 5.1 software program. All images taken were averages of 10 scans.

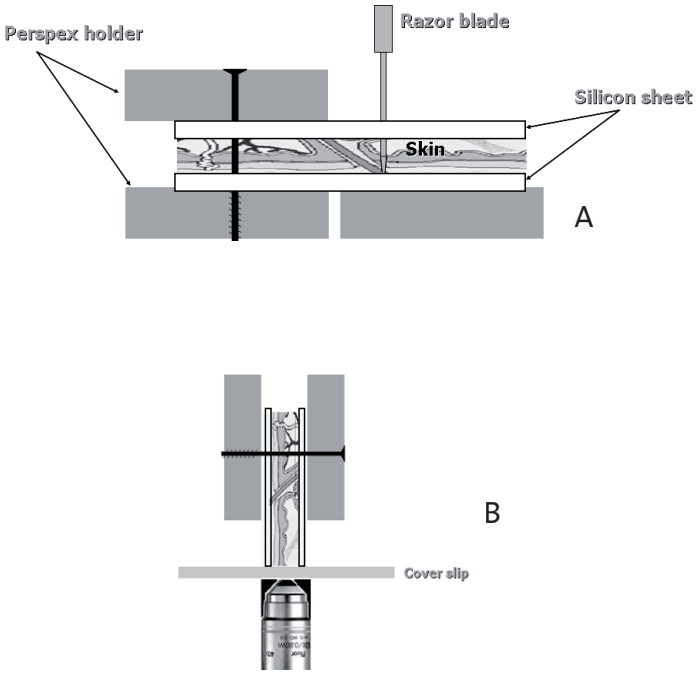

Figure 8.3. Diagram of the cutting device used to obtain a cross-section of the skin for CSLM imaging. After the penetration experiment, a square piece from the skin diffusion area, was pinched between two silicon sheets and fixed in perplex holders as shown in A. After cutting the skin (sandwiched between the silicon) perpendicular from dermis to SC, it was mounted in a sample holder as shown in B.

Statistical analysis

For statistical analysis the independent student-*t*-test was applied (GraphPad Prism 3.02) using critical level of significance of 0.05 (*P* values gives are 2-tailed).

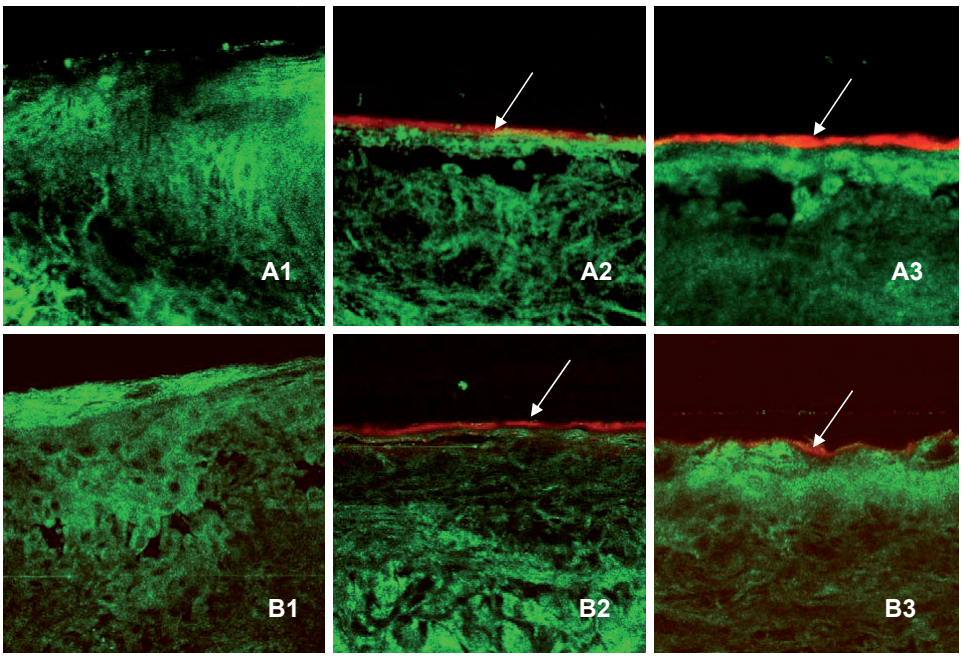

Figure 8.4. CSLM images of cross-sections of human skin after the skin penetration experiment at pH 7.4 (series A) and pH 5.2 (series B). PBS, pH 7.4 (A1), 80 µM Sylsens B in PBS (A2), 160 µM Sylsens B in PBS (A3), 5 mM citric acid, pH 5.2 (B1), 80 µM Sylsens B in 5 mM citric acid, pH 5.2 (B2) and 160 µM Sylsens B in 5 mM citric acid, pH 5.2 (B3). Arrows indicate the fluorescence of Sylsens B. Magnifications used: 400 X.

RESULTS

Sylsens B does not penetrate dermatomed human skin

When using dermatomed human skin we observed no penetration of Sylsens B even when we applied the highest concentration of this photosensitizer (160 µM) at both pH 7.4 and 5.2 (see Table 1). The results show that the fluorescence intensity measured in the acceptor chamber after 24 hours application of Sylsens B at pH 7.4 did not differ significantly from the control. At pH 5.2, similar results were obtained. In our CSLM experiments the absence of Sylsens B in the viable epidermis and dermis was evident (Fig. 8.4). The SC, the viable epidermis and the dermis are visible due to the green autofluorescence at 514 nm (see Fig. 8.4 A1 and B1 for 7.4 and 5.2 respectively). It

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could be noticed that at both pH, the orange fluorescence of Sylsens B was localized only on the surface and did not penetrate into the deeper skin layers.

Donor Chamber Sylsens B concentration (µg/ml)	*Acceptor Chamber Fluorescence[a] (AU)*	
	pH 7.4	*pH 5.2*
0	8.6 ± 0.7	11 ± 3
62	11 ± 2	11 ± 3
123	11 ± 3	12 ± 3

Table 8.1. *In vitro* penetration behavior of Sylsens B through human dermatomed skin at pH 7.4 (PBS) and 5.2 (5 mM citric acid/sodium citrate buffer).
[a] The accumulated amount of fluorescence (in AU) measured in the acceptor liquid after 24 hours at 657 nm upon excitation at 424 nm. Results as given mean ± standard deviations

A skin barrier damaged by fungal growth causes Sylsens B penetration at pH 7.4, but not at pH 5.2.

The influence of preceding fungal growth on the Sylsens B penetration through SC was investigated on SC sheets infected with *T. rubrum*. The sheets were used at two different time points after the spore inoculation. The results are provided in the Table 8.2 (pH 7.4) and Table 8.3 (pH 5.2).

Donor Chamber	*Acceptor Chamber*			
	3 days fungal growth		*5 days fungal growth*	
Test condition	*Fluorescence (AU)*	*[Sylsens B] (µg/ml)*	*Fluorescence (AU)*	*[Sylsens B] (µg/ml)*
PBS, pH 7.4 (no fungus)	8 ± 3	n.p	10 ± 1	n.p
Sylsens B (123 µg/ml) in PBS, pH 7.4 (no fungus)	12 ± 3	n.p	13 ± 3	n.p
PBS, pH 7.4 (fungus present)	9 ± 2	n.p	12 ± 2	n.p
Sylsens B (123 µg/ml) in PBS, pH 7.4 (fungus present)	10 ± 1	n.p	64 ± 12[a]	0.074 ± 0.024[a]

Table 8.2. *In vitro* penetration behavior of Sylsens B through SC infected with *T. rubrum* at pH 7.4.
Studies were performed at 3 and 5 days after the *T. rubrum* spore inoculation. The accumulated amount of fluorescence (in AU) measured in the acceptor liquid after 24 hours at 657 nm upon excitation at 424 nm. Results as given mean ± standard deviations.
[a] Significantly different compared to uninfected SC ($t(10) = 8.034$, $P = 0.0002$) and to the application of PBS ($t(10) = 8.310$, $P = 0.0002$).
n.p: no Sylsens B present.

The results obtained at pH 7.4 showed that there was no Sylsens B penetration through uninfected SC (control) and SC three days after microconidia inoculation. However, 5 days after inoculation, a considerable increase in fluorescence was detected in the acceptor fluid, which was significant different from uninfected SC. Also the application of Sylsens B to infected SC resulted in a significantly higher fluorescence in the accepter phase when compared to the application of the control PBS in the presence and absence of fungus growth.

Donor Chamber	***Acceptor Chamber***			
Test condition	**3 *days fungal growth***		**5 *days fungal growth***	
	Fluorescence (AU)	***[Sylsens B] (µg/ml)***	***Fluorescence (AU)***	***[Sylsens B] (µg/ml)***
Citric acid/sodium citrate, pH 5.2 (no fungus)	7 ± 3	n.p	6 ± 2	n.p
Sylsens B (123 µg/ml) in Citric acid/sodium citrate, pH 5.2 (no fungus)	10 ± 3	n.p	7.8 ± 0.7[a]	0.0021 ± 0.0008[a]
Citric acid/sodium citrate, pH 5.2 (fungus present)	6 ± 2	n.p	8 ± 2	n.p
Sylsens B (123 µg/ml) in Citric acid/sodium citrate, pH 5.2 (fungus present)	8 ± 2	n.p	12 ± 5	n.p

Table 8.3. *In vitro* penetration behavior of Sylsens B through SC infected with *T. rubrum* at pH 5.2.
Studies were performed at 3 and 5 days after the *T. rubrum* spore inoculation. The accumulated amount of fluorescence (in AU) measured in the acceptor liquid after 24 hours at 657 nm upon excitation at 424 nm. Results as given mean ± standard deviations.
[a] Significantly different ($t(10) = 2.482$, $P = 0.0324$) from the control with the calculated amount of Sylsens B penetrated (µg Sylsens B/ml acceptor liquid).
n.p: no Sylsens B present.

When pH 5.2 was used, similar results were obtained with the exception of the experiments using SC five days after spore inoculation. In this case, no penetration of Sylsens B was observed at pH 5.2 in contrast to the situation when the photosensitizer was dissolved in pH 7.4 (compare Table 8.2 to 8.3). Even when the highest concentration of Sylsens B was applied there was no statistically significant difference with controls. However, when the SC was pre-treated for 24 hours with 1% SLS, a significant amount of Sylsens B (applied at pH 5.2) was detected in the acceptor phase (see Table 8.4).

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Donor Chamber	*Acceptor Chamber*	
Test condition	*Fluorescence (AU)*	*[Sylsens B] (µg/ml)*
Citric acid/sodium citrate, pH 5.2 (normal SC)	7 ± 3	n.p
Sylsens B (123 µg/ml) in Citric acid/ sodium citrate, pH 5.2 (normal SC)	5 ± 1	n.p
Citric acid/sodium citrate, pH 5.2 (SC pre-treated with SLS)	5 ± 2	n.p
Sylsens B (123 µg/ml) in Citric acid/sodium citrate, pH 5.2 (SC pre-treated with SLS)	124 ± 109[a]	0.10 ± 0.08[a]

Table 8.4. *In vitro* penetration behavior of Sylsens B through SC pre-treated (24 hours) with 1 % SLS at pH 5.2.
The accumulated amount of fluorescence (in AU) measured in the acceptor liquid after 24 hours at 657 nm upon excitation at 424 nm. Results as given mean ± standard deviations.
[a] Significantly different compared to sodium citric acid buffer $t(9) = 2.473$, $P = 0.0354$ and compared to untreated SC $t(9) = 2.486$, $P = 0.0347$.
n.p: no Sylsens B present.

DISCUSSION

The main goal of this study was to examine the skin penetration behaviour of a previously successful Sylsens B formulation (pH 5.2) that could be used in future clinical PDT experiments. Next to the healthy dermatomed skin, we used isolated SC because the SC forms the main skin barrier for penetration. (20-23)
We observed no penetration in healthy skin of 160 µM solution of Sylsens B neither at pH 7.4 nor 5.2 with 160 µM Sylsens B. The positive charge of Sylsens B, the hydrophilic character and the relatively high molecular weight might contribute to these findings. (24,25) However, when the SC was infected with *T. rubrum* we observed some differences in Sylsens B penetration between pH 5.2 and pH 7.4. The 5-day fungal growth resulted in Sylsens B penetration at pH 7.4, but not at pH 5.2.
We demonstrated in our previous work that at pH 5.2 a selective binding of this photosensitizer to the fungus occurred. Apparently, the presence of fungal particles in SC offers an increased binding area for Sylsens B at pH 5.2 and there is no Sylsens B available to penetrate the damaged skin. At pH 7.4, the binding capacity of Sylsens B to fungal elements is lower and this makes the penetration of Sylsens B molecules possible. (12) Although the amount of Sylsens B that penetrated the skin barrier at pH 7.4 was calculated to be 0.1% of the applied concentration, this amount could still potentially affect the underlying healthy epidermal and dermal cell layers.

From these experiments we conclude that Sylsens B in the water solution with low ion concentration and pH 5.2 does not penetrate the stratum corneum, even in the presence of *T. rubrum*). The risk of a systemic effect of Sylsens B is therefore minimized and from this point of view this formulation seems to be safe for a future application in a clinical setting.

However, when the SC was pretreated with SLS, Sylsens B could easily penetrate even at pH 5.2. In this case, the skin barrier was strongly reduced and the binding groups for Sylsens B were missing because of the absence of fungal elements. Therefore, the use of a detergent like SLS before PDT with Sylsens B should be avoided.

ACKNOWLEDGEMENTS

We thank Dr. Rob van der Steen from Buchem Holding BV (Lieren, The Netherlands) for the synthesis of Sylsens B and for his valuable advice regarding the porphyrin chemistry. We are also grateful to Stefan Romijn (Drugs delivery Technology Department, Leiden University, The Netherlands) for providing us with the images of Figure 8.2.

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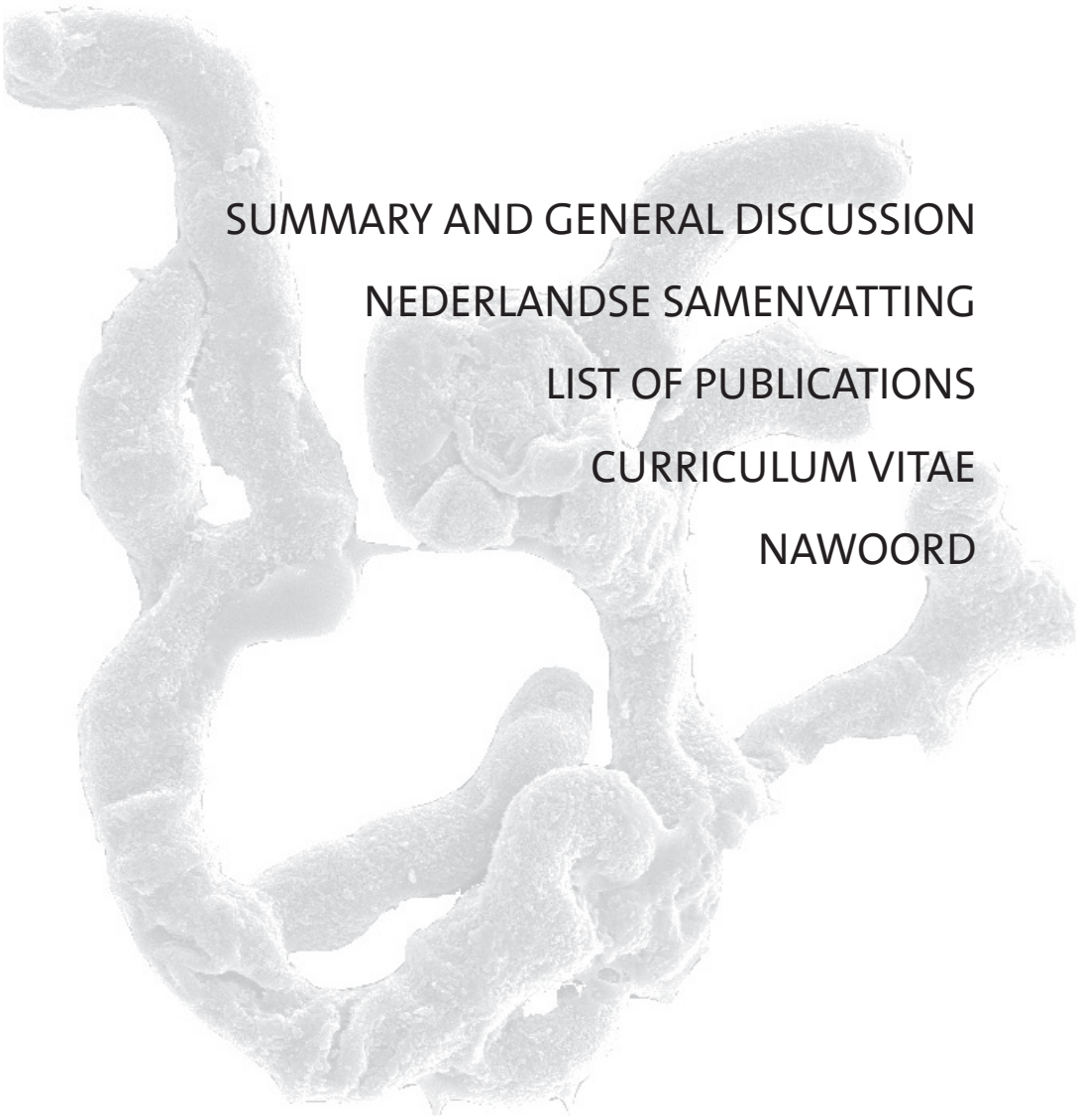
Chapter IX



SUMMARY AND GENERAL DISCUSSION

NEDERLANDSE SAMENVATTING

LIST OF PUBLICATIONS

CURRICULUM VITAE

NAWOORD

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

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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.

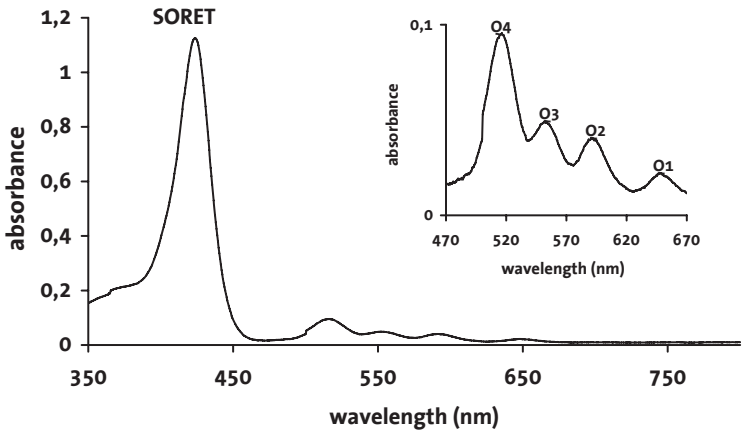

Figure 9.1. Absorption spectrum of Sylsens B in methanol.

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NEDERLANDSE SAMENVATTING

Het onderzoek beschreven in dit proefschrift heeft als doel huidinfecties veroorzaakt door de huidschimmel *Trichophyton rubrum* (*T. rubrum*) éénmalig effectief te behandelen met behulp van een fotodynamische behandeling (PDT).

PDT heeft betrekking op een behandeling, binnen een biologisch systeem, met een lichtgevoelige stof, de fotosensibilisator, in combinatie met licht en zuurstof en berust op het volgende principe. In aanwezigheid van licht van een geschikte golflengte zal de fotosensibilisator in staat zijn tot absorptie van dit licht en komt hierdoor op een hoger energieniveau terecht. De op deze wijze verkregen energie kan op verschillende manieren door de fotosensibilisator benut worden. Het optreden van een chemische reactie behoort tot de mogelijkheden. Hierbij geeft het aangeslagen molecuul zijn energie door aan een ander molecuul. In een biologisch systeem is dit meestal moleculaire zuurstof en kunnen twee verschillende typen reacties ontstaan, beiden leidend tot biologische schade. Genoemde reacties staan bekend als *type I* en *type II* reactie. In een *type I* reactie betreft het een overdracht van elektronen of waterstof, terwijl in een *type II* reactie een energie overdracht plaatsvindt waarbij een reactief kortlevend zuurstofdeeltje, singlet zuurstof ($^1O_2$) ontstaat.

Door het toenemende optreden van bacteriële resistentie voor antibiotica, zou het gebruik van PDT een goed alternatief kunnen zijn voor de behandeling van gelokaliseerde huidinfecties. Ook de behandeling van wondinfecties zou hieronder kunnen vallen. Op dit moment is het bekend dat bij fotodynamische behandeling van micro-organismen $^1O_2$ verantwoordelijk is voor schade. Doordat in een biologische omgeving de levensduur van $^1O_2$ slechts 100 tot 250 ns bedraagt, zal de plaats van $^1O_2$ productie in biologische systeem bepalend zijn voor de plaats van het schadelijke effect. Cruciaal voor het welslagen van een fotodynamische behandeling is de binding van de fotosensibilisator aan het biologische doel.

Binnen dermatologie is het gebruik van PDT uitgebreid onderzocht, echter nog zeer beperkt voor de behandeling van oppervlakkige huidschimmelinfecties. Huidschimmels leven niet alleen in de huid maar komen ook voor in haren en nagels. Al deze weefsels bezitten namelijk keratine. Het is dit keratine waarop een huidschimmel in staat is te groeien en daarmee een infectie te veroorzaken. Deze infecties staan ook wel bekend onder de naam tinea, met daarachter een verwijzing naar het lichaamsdeel waar de infectie zich bevindt.

Tot op heden zijn er verschillende huidschimmels onderworpen aan PDT waarbij

verschillende fotosensibilisatoren werden uitgetest, zoals onder andere methyleen blauw en phthalocyanine derivaten. Een complete schimmel dood werd echter niet aangetoond.
In dit onderzoek wordt als fotosensibilisator gebruik gemaakt van gesynthetiseerde chemische verbindingen, bekend onder de verzamelnaam porfyrines. Het onderzoek focusseert zich verder op de meest hardnekkig te behandelen dermatophyte, *T. rubrum*.

T. rubrum gekweekt in suspensie blijkt gevoelig voor PDT met porfyrine fotosensibilisatoren (hoofdstuk 2)

Gezien de beperkingen van de huidige beschikbare behandelingsmethoden voor tinea, met name indien veroorzaakt door *T. rubrum,* is onderzoek gestart naar een mogelijke gevoeligheid van *T. rubrum* voor PDT.
T. rubrum werd hiertoe in een vloeibaar medium gekweekt en als zodanig behandeld met verschillende porfyrine fotosensibilisatoren in combinatie met zichtbaar licht. Het aldus gevonden PDT effect werd vergeleken met het effect gevonden voor andere, klassieke fotosensibilisatoren.
Voor deze laatste categorie fotosensibilisatoren werd slechts een groeivertragend effect waargenomen, terwijl voor met name twee porfyrines, Sylsens B en DP mme, volledige schimmeldood werd verkregen.

Het gebruik van rood licht blijkt effectief te zijn als onderdeel van een porfyrine PDT van T. rubrum : zowel mycelium als sporen blijken gevoelig voor de behandeling (hoofdstuk 3)

Aangezien huidschimmels ook vaak te vinden zijn in de diepere haarfollikels zouden fotosensibilisatoren die gebruik kunnen maken van rood licht aan te bevelen zijn. Rood licht is in staat om dieper in de huid door te dringen en dus ook in de haarfollikels. Bovendien zou het gebruik van rood licht bij succes een goed perspectief kunnen bieden voor de fotodynamische behandeling van de veel voorkomende schimmelinfectie van de nagel (bekend als onychomycose). Deze wordt meestal veroorzaakt door *T. rubrum*.
Bij deze studie werden suspensies van de schimmel en zijn sporen (de microconidia) behandeld met PDT met rood licht. Sylsens B en DP mme werden hierbij als fotosensibilisator gebruikt en het verkregen resultaat werd vergeleken met het resultaat gevonden met een nieuw gesynthetiseerde porpfyrine verbinding, QDD. Deze

laatste porfyrine verbinding is in staat om meer rood licht te absorberen dan Sylsens B en DP mme en zou theoretisch dus met rood licht een hoge(re) PDT effectiviteit moeten geven.
Dit werd echter niet gevonden; QDD gaf slechts een groeivertragend effect. Voor dit groeivertragende effect bleek ook nog eens een veel hogere concentratie (10 maal zo hoog) nodig te zijn dan tot nu toe gebruikelijk voor Sylsens B. Voor deze laatste verbinding en DP mme werd wel ten opzichte van zowel schimmel mycelium als microconidia (beide getest in oplossing) een volledig dodend effect aangetoond.
Vermoedelijk speelt bij het verkregen resultaat de aangetoonde foto-instabiliteit van QDD een geringe rol. Hierbij zal deze verbinding bij belichting zelf schade ondervinden en daardoor steeds minder licht kunnen absorberen. Aannemelijker is in dit geval echter het ontbreken van een geschikte bindingscapaciteit van het neutrale QDD molecule. Zowel Sylsens B als DP mme zijn geladen moleculen. Sylsens B heeft een positieve lading en DP mme een negatieve. Dit maakt het voor deze verbindingen makkelijk mogelijk om bijvoorbeeld aan de schimmelwand te binden.

Ontwikkeling van een nieuw ex vivo model om de PDT effectiviteit van porfyrines te testen ten opzichte van T. rubrum groeiend op geïsoleerde humaan huidoppervlak (hoofdstuk 4)

Door de verkregen succesvolle resultaten voor de PDT met Sylsens B en DP mme van *T. rubrum* gekweekt in een vloeibaar medium hebben wij ons de vraag gesteld in hoeverre dit resultaat ook bereikt zou kunnen worden in een situatie vergelijkbaar met de klinische situatie.
Om hier achter te komen werd een *ex vivo* model ontwikkeld. In dit model werd gebruik gemaakt van het hoornlaagje van humane huid, het stratum corneum (SC). Door op dit stukje SC de sporen van *T. rubrum* te enten en deze uit te laten groeien bij 28°C ontstaat een situatie die lijkt op de klinische situatie.
De opzet van de experimenten met fotosensibilisatoren was zodanig dat de fotodynamische behandeling met rood licht plaatsvond op verschillende tijdstippen na enting van de schimmel op het SC.
Het eerste in het oog springende resultaat bleek de geringere gevoeligheid van *T. rubrum* voor PDT onder deze *ex vivo* omstandigheden. Om hetzelfde schimmeldodend effect te verkrijgen bleek een veel hogere fotosensilbilisator concentratie nodig te zijn. De aanhechting van de schimmel aan huid, een mechanisch proces wat een rol speelt bij het ziekte proces in een klinische situatie, zou een rol kunnen spelen in de lagere

gevoeligheid van de schimmel voor de PDT. We vonden dat kort (8 uur) na enting de gevoeligheid voor PDT hoger was dan wanneer de schimmel op een later tijdstip (bijvoorbeeld 72 uur na enting) werd behandeld met PDT. Het stadium waarin zich nog sporen al dan niet ontkiemd bevonden (8 uur na enting) bleek het meest gevoelig te zijn voor PDT. Latere stadia (getest werd tot 72 uur na enting) waarbij voornamelijk volledig ontwikkeld mycelium aanwezig is bleken moeilijker te behandelen.
Het medium waarin (voorafgaand aan de belichtingsperiode) incubatie plaatsvond van het geïnfecteerde SC met fotosensibilisator bleek een opmerkelijke rol te spelen. Maakten we gebruik van Dulbecco's modified Eagle medium (DMEM) dan bleek de PDT activiteit van Sylsens B en DP mme vergelijkbaar. Vervanging van het DMEM medium door gedestilleerd water bleek de PDT effectiviteit van Sylsens B te verhogen en die van DP mme te verlagen. Factoren zoals de pH van het water en het ontbreken van een groot aantal ionen in het water zouden hiervoor verantwoordelijk kunnen zijn.
Toepassing van fluorescentie microscopie toonde aanwezigheid van Sylsens B aan de buitenkant van de schimmelwand in geval van een donker en niet succesvolle fotodynamische behandeling. Na een effectieve PDT bleek Sylsens B evens binnen de schimmelwand aantoonbaar.
Belangrijke conclusies uit dit onderzoek waren de afhankelijkheid van de PDT gevoeligheid van het *T. rubrum* groeistadium.

Lage ionensterkte en pH blijken de PDT effectiviteit van de positief geladen fotosensibilisator Sylsens B te verhogen (hoofdstuk 5)

In dit gedeelte van de studie hebben we geprobeerd om een verklaring te vinden voor de gevonden resultaten en geprobeerd het mechanisme te ontrafelen verantwoordelijk voor de PDT van *T. rubrum* met rood licht in de *ex vivo* situatie.
Hierbij werd een groot aantal factoren onderzocht die van invloed zouden kunnen zijn op de PDT met rood licht van *T. rubrum*, aangehecht aan humaan SC. Belangrijkste onderzochte factoren waren de pH en ionensterkte van het incubatiemedium, fotochemische eigenschappen van de fotosensibilisatoren en de invloed van een keratinase remmer. Keratinase is een enzym dat door deze schimmel in grote hoeveelheid wordt gemaakt indien de schimmel uitsluitend keratine als voedingsbron tot zijn beschikking heeft. Derhalve speelt de productie van dit enzym in de voortgang van het ziekteproces in een klinische situatie een belangrijke rol. In deze situatie schakelt de schimmel, na aanhechting op de huid, over op een in de huid aanwezige voedingsbron (het keratine) door de productie van keratinase. Verloopt dit proces

goed dan is de infectie een feit.
De positief geladen verbinding Sylsens B bleek, getest op 3 verschillende schimmel stadia (van spore tot mycelium), het beste resultaat te geven bij een lage ionensterkte en pH van 5.2. Voor alle geteste groeistadia werd een volledige dood van de schimmel verkregen, echter in geval van behandeling van het mycelium (72 uur na enting op het SC) werd schimmeldood in slechts 80 tot 90 % van de gevallen aangetoond.
Belangrijk bij de verklaring voor de gevonden resultaten is de mogelijkheid van Sylsens B om door zijn positieve lading bij de pH van 5.2 selectief te kunnen binden aan de schimmel en minder tot niet aan de huid zelf. Dit komt doordat wij in dit stukje onderzoek het bewijs hebben geleverd voor het feit dat er een klein pH gebied (tussen pH 3.5 en 5.5) is waarin de schimmel negatief en het SC meer neutraal geladen is.
Andere interessante uitkomsten uit dit onderzoek bleken het PDT effectiviteit verhogende effect van de keratinase remmer alsmede het aantonen van een pH effect binnen de factor groeistadium en een groeistadium effect binnen de factor pH.

PDT met een dodelijke Sylsens B concentratie leidt tot grote veranderingen in de morfologie van de schimmelwand (hoodstuk 6)
Er is weinig onderzoek gedaan naar het effect van PDT op de morfologie van micro-organismen. Dit geldt zeker voor de schimmel *T. rubrum*.
Derhalve hebben wij een scanning elektronen microscopisch (SEM) onderzoek opgezet om inzicht te krijgen in het effect van een dodelijke dosis Sylsens B op de morfologie van de schimmel wand. Analoog aan ons eerdere onderzoek hebben we effecten bekeken in verschillende groeistadia van de schimmel en op verschillende tijdstippen na de behandeling. Om een zo volledig mogelijk beeld te krijgen werd ook gekeken naar het effect van de effectieve dosis van rood licht alleen en naar het effect van Sylsens B in het donker.
Sylsens B zonder licht applicatie bleek duidelijk morfologische veranderingen teweeg te brengen, waaronder zwellingen en deuken zichtbaar op de schimmelwand. Echter slechts een effectieve PDT resulteerde in dusdanige morfologische schimmelwand veranderingen dat de schimmel dit niet kon overleven. Hierbij bleek uiteindelijk de schimmelwand volledig beroofd te zijn van zijn karakteristieke morfologie en uiteindelijk resulteerde de behandeling in leeggelopen platte schimmel elementen. De effecten bleken reeds zeer kort na de behandeling waarneembaar.
Bij een niet geheel effectieve PDT werd hernieuwde schimmelgroei zichtbaar op bepaalde plaatsen in een verder dode schimmel. Dit bleek voornamelijk aan de

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toppen van de hyphen het geval te zijn. Voor deze hyphen toppen bleek onder normale omstandigheden in het groeistadium corresponderend met 72 uur na enting (het lastigst te behandelen stadium) een afwijkende morfologie gevonden. Het zou kunnen dat hierdoor Sylsens B minder goed aan deze toppen kan binden en daardoor dus de PDT effectiviteit juist op deze plekken geringer is.

Ontwikkeling van een testsysteem voor het testen op aanwezigheid van fotomutageniciteit (hoofdstuk 7)

Door het toenemend gebruik van fotosensibilisatoren voor medische applicaties zou een goed testsysteem voor mogelijke mutagene potentie van fotosensibilisatoren noodzakelijk zijn. Hoewel er een groot aantal testsystemen voor mutageniciteit zijn en deze veelal ook zijn aangepast voor het gebruik van licht binnen het systeem, houden zij alle toch bepaalde beperkingen.

Gebaseerd op een gevalideerd testsysteem, bekend als SMART, werd een systeem ontwikkeld voor de detectie van fotochemische genotoxiciteit. Hierbij werd methlyleen blauw, waarvan bekend is dat het fotomutageniciteit kan induceren, gekozen als positieve controle.

Binnen het door ons opgezette systeem, de Photo-SMART, werd voor de porfyrine Sylsens B onder de gegeven testomstandigheden een indicatie gevonden voor mutageniciteit, terwijl voor een andere porfyrine verbinding, hematoporfyrine, fotomutageniciteit afwezig bleek te zijn.

De positief geladen porfyrine fotosensibilisator Sylsens B penetreert niet door humane huid (hoofdstuk 8)

De huidige behandelingsmethoden voor tinea hebben toch vaak nog nadelen, voornamelijk het terugkeren van de infectie en hierdoor de lange duur van de behandeling worden als zeer vervelend ervaren.

Een klinische toepassing van PDT voor tinea zou mogelijk een waardevolle toevoeging kunnen zijn op bestaande behandelingen.

Voor gebruik van Sylsens B in een PDT applicatie is het niet nodig en zeker niet wenselijk dat Sylsens B door het huidoppervlak penetreert naar dieper gelegen huiddelen.

Derhalve werd het penetratiegedrag van Sylsens B onderzocht onder verschillende omstandigheden. Zo werd gebruik gemaakt van gezonde huid, van SC geïnfecteerd met *T. rubrum* alsmede van chemisch beschadigd SC. De invloed van pH 5.2 in een voor PDT effectieve formulering (lage ionensterkte) met Sylsens B werd bekeken en

vergeleken met Sylsen B in een fosfaat gebufferde saline oplossing van pH 7.4. Deze laatste oplossing bezit een hoge ionensterkte.
Bij gezonde huid bleek er, zowel bij de pH 5.2 als de pH 7.4 formulering geen Sylsens B penetratie plaats te vinden. Beschadiging van het huidoppervlak door *T. rubrum* groei gaf bij de formulering van pH 7.4 aanleiding tot geringe Sylsens B penetratie, maar niet bij gebruik van onze succesvolle PDT formulering.

Slotopmerkingen en de toekomst

Dit onderzoek heeft geleid tot het tot stand komen van een formulering die gebruikt kan worden om binnen één enkele fotodynamische behandeling tinea veroorzaakt door *T. rubrum* effectief te bestrijden. Deze formulering zou bij voorkeur moeten bestaan uit de positief geladen fotosensibilisator Sylsens B in een waterige oplossing met een pH waarde liggend tussen 4.5 en 5.7 (gepatenteerd pH gebied).
Met deze formulering is het mogelijk om de hardnekkige veroorzaker van tinea, *T. rubrum* , uit te schakelen door PDT met rood licht. Opmerkelijk hierbij is het sterke effect van deze behandeling op de schimmelsporen. Dit in tegenstelling tot het effect van de meeste huidige behandelingsmethodieken. Deze hebben veelal uitsluitend effect op de metabolisch actieve vorm van de schimmel, waarbij de sporen dan ongemoeid blijven. Indien deze in de huid achterblijven kunnen zij daar opnieuw een infectie starten.
Voor de verklaring van het grotere PDT effect met Sylsens B op de sporen van *T. rubrum* in vergelijking met het mycelium, zou het verschil in grootte in rol kunnen spelen. Aangezien binding noodzakelijk is voor het welslagen van PDT zou het zo kunnen zijn dat het totale mycelium oppervlak te groot is voor een effectieve fotosensibilisator binding. Voor het kleinere sporenoppervlak zou dit dan minder een probleem zijn. Daarnaast zou de hogere pigmentering van sporen ten opzichte van mycelium ook een rol kunnen spelen. Gedacht wordt hierbij aan de aanwezigheid van lichtabsorberende stoffen aanwezig op het oppervlak van sporen maar weer niet op dat van mycelium.Verder heeft de sporenwand ook een uniforme morfologie. Hierdoor zal overal dezelfde, effectieve bindingscapaciteit voor Sylsens B aanwezig zijn. De dikkere sporenwand doet duidelijk niet ter zake bij de hogere gevoeligheid voor PDT. Dit komt omdat Sylsens B bindt aan de buitenkant van de schimmelwand.
Het mycelium van *T. rubrum* , 72 uur na enting, is het minst gevoelig gebleken voor de PDT met 160 µM Sylsens B en rood licht. Ook hier konden we een volledige dood van de schimmel bewerkstelligen, maar dit bleek slechts in 80 tot 90% het geval te zijn.

In de andere gevallen gaf deze PDT aanleiding tot een groeivertraging en zagen we op bepaalde plaatsen in de hyphen hernieuwde schimmelgroei optreden. Veelal bleek dit het geval te zijn bij de toppen van de hyphen. Bij gezonde hyphen bleek, met behulp van SEM, dat de toppen duidelijk een afwijkende morfologie bezaten in vergelijking met andere delen van de hyphen (waargenomen op 72 uur na enting). Het zou kunnen zijn dat het juist die afwijkende morfologie is die ervoor zorgt dat Sylsens B minder goed kan binden aan deze toppen. Hierdoor zouden deze delen minder gevoelig kunnen zijn voor PDT met Sylsens B. Dit maakt hergroei ter plekke mogelijk.

Om toch in alle 100% van de behandelingen een volledige schimmeldood te krijgen zou toevoeging van de keratinase remmer tijdens incubatie van *T. rubrum* met Sylsens B en oplossing kunnen bieden. Ook de hele PDT behandeling binnen 48 uur herhalen zou volgens de resultaten van het SEM onderzoek moeten leiden tot 100% volledige dood van de schimmel. De gedachtegang hierachter is dat uit de SEM studie duidelijk werd dat de gevonden afwijkende morfologie aan de toppen van de hyphen niet eerder zichtbaar was dan 72 uur na enting. Daarvoor bleken geen grote verschillen waarneembaar tussen de toppen en andere plekken in de hyphen. Recentelijk onderzoek wees uit dat inderdaad bij herhaling van de PDT met 160 µM Sylsens B binnen 17 uur de 100% gehaald werd.

Wanneer we onze resultaten vergelijken met de literatuur bestaande over PDT van micro-organismen dan kunnen we zeggen dat ook bij deze schimmel de PDT beter werkt met een positief geladen fotosensibilisator dan met een negatief geladen en beiden werken weer beter dan een neutrale fotosensibilisator.

Binding van de fotosensibilisator, Sylsens B, aan de schimmelwand veroorzaakt een destabilisatie van de wand waardoor de schimmel al wat gevoeliger wordt voor de PDT en de productie van $^1O_2$. Door deze PDT treedt zodanige schade op aan de schimmelwand dat lekkages optreden. Op dat moment zal Sylsens B naar binnentreden en zal gedurende verdere belichting op nog meer plaatsten (ook binnenin de schimmel) schade plaatsvinden ten gevolge van productie van $^1O_2$.

Deze binding van Sylsens B met componenten aanwezig in het buitenste deel van de schimmelwand berust vermoedelijk op elektrostatische interacties tussen tegengestelde ladingen. In dit onderzoek is niet ingegaan op de aard van de bindingsplaats voor Sylsens B op de schimmelwand. Het vermoeden bestaat echter dat mogelijk negatief geladen fosforeenheden aanwezig zijn in de buitenste laag van de schimmelwand. De aanwezigheid van dergelijke negatief geladen groepen, waar ook AB aan bindt, is voor ander schimmels aangetoond. Wij hebben aangetoond dat

binding van AB aan *T. rubrum* de binding van Sylsens B verminderde.
Tot slot is het belangrijk te vermelden dat dit onderzoek heeft aangetoond dat door het bewerkstelligen van een selectieve binding van Sylsens B aan *T. rubrum* (gebruikmakend van de formulering met pH 5.2 en lage ionensterkte) niet alleen penetratie van Sylsens B naar dieper gelegen delen van de huid wordt voorkomen, maar hierdoor tevens gezonde oppervlakkige huidlagen beschermd worden tegen ongewenste PDT effecten.
Voor de nabije toekomst staan op de eerste plaats klinische PDT studies gepland met Sylsens B als fotosensibilisator in een waterige formulering van pH 5. Met dit doel voor ogen moet zo veel mogelijk gestreefd worden naar 100% schimmel dood in alle gevallen. Zoals aan aangegeven zou dit bereikt kunnen worden door aan de formulering de keratinase remmer toe te voegen of de gehele behandeling binnen 17 uur te herhalen. Beide opties zijn in de praktijk niet aan te bevelen. Beter is het om te kijken in hoeverre het gebruik van het licht en de lichtbron geoptimaliseerd kunnen worden. Sylsens B absorbeert slechts zeer gering in het golflengtegebied van het rode licht zoals dat hier gebruikt wordt. Hoewel dit toch leidt tot effectiviteit is het denkbaar dat wanneer er licht gebruikt zou worden waarin Sylsens B een hogere absorptie heeft, dit tot nog hogere effectiviteit zou kunnen leiden. Het is bekend dat Sylsens B beter absorbeert in het blauwe gedeelte van het spectrum (424 nm) en in het begin van het golflengtegebied van het groene licht (514 nm). Toepassing van deze golflengtegebieden zou niet alleen kunnen leiden tot de noodzakelijke 100% volledige schimmeldood in alle gevallen maar ook de hiervoor benodigde Sylsens B concentratie kunnen verlagen. Gebruik van groen licht heeft als additioneel voordeel dat het in staat is om door de nagel heen te gaan, net als het geval is voor rood licht. Dit is uitermate belangrijk voor het toekomstig onderzoek waarbij we gebruik willen maken van nieuw ontwikkelde multifunctionele porfyrine fotosensibilisatoren, structuurverwant aan Sylsens B. Dit onderzoek moet zich gaan richten op PDT van een schimmelinfecties van huid, haren en nagels veroorzaakt door *T. rubrum* en andere dermatophyten.

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Publication number and date: US2006258635, 2006-11-16
PCT number: PCT/NL04/00079
Priority numbers: NL20031022597 20030205; WO2004NL00079 20040205

"A pharmaceutical composition for the treatment of a fungal skin disorder and a method for the preparation thereof"
Smiijs, Geertruida MT (NL, first inventor) and Schuitmaker, Johannes J (NL)
Publication number and date: WO2008/026915, 2008-03-06
PCT number: PCT/NL2007/000211
Priority number: NL1032375

CURRICULUM VITAE

Threes Smijs werd geboren op 23 oktober 1954 te Breda. Haar middelbare schoolopleiding genoot zij aan het Thomas More College in Den Haag. Na haar eindexamen HAVO in 1972 begon zij aan een HBO-B opleiding Biochemie aan de Delftse Analisten Cursus (later het Van Leeuwenhoek Instituut) te Delft. Eind 1974 vertrok zij naar Jakarta en maakte zich de resterende vakken van de analistenopleiding door zelfstudie eigen, resulterend in 1977 in een theorieverklaring HBO-B Biochemie. In Jakarta werkte zij tot aan haar terugkeer naar Nederland in 1978 aan de medische faculteit van de katholieke universiteit Atma Jaya, waar zij een drinkwateronderzoek opzette en uitvoerde en microbiologie doceerde.
Na haar HBO stage aan de Technische Hogeschool te Delft waar zij onderzoek verrichte aan methanol dehydrogenase uit *Hyphomicrobium X,* ontving zij in 1979 het diploma HBO-B Biochemie.
In datzelfde jaar begon zij bij Academisch Ziekenhuis Leiden als onderzoeksanaliste bij een onderzoek dat deel uitmaakte van het promotie traject van P.J. van Bladeren onder leiding van prof. dr. D.D. Breimer en prof. dr. A. van der Gen en dr. G. R. Mohn. Dit onderzoek had betrekking op de dubbele rol van glutathion conjugatie bij de biotransformatie van xenobiotica. Als extraneus schreef zij zich in datzelfde jaar in voor de studie scheikunde aan de Universiteit Leiden en behaalde een theorieverklaring kandidaats chemie en een voltooid bijvak farmacologie, waarbij onderzoek werd verricht naar de stereoselektiviteit van de conjugatie van (+) en (-) styreenoxide met glutathion *in vitro* en de mutageniciteit van styreenoxide voor *Salmonella thyphimurium* bacteriën.
Eind 1994 startte zij met de studie WO-Milieu-natuurwetenschappen aan de Open Universiteit Nederland (OUNL) en in 2000 keerde zij terug naar wetenschappelijk onderzoek bij het Leids Universitair Medisch Centrum (LUMC). Van 2000 tot 2004 werkte zij als research analiste op een STW-project (STW LKG 4890) bij de afdeling oogheelkunde. In dit project werd, in samenwerking met prof. dr. J. Lugtenburg van de Universiteit Leiden onderzoek verricht naar een fotodynamische kankertherapie gebaseerd op gesynthetiseerde porfyrines. Tegelijkertijd zette zij haar afstudeeronderzoek naar een milieuvriendelijk (porfyrine) insecticide op. Dit onderzoek werd onder leiding van dr. G.E.I. Holtkamp, dr. E.J. Middelbeek en dr. J.J. Schuitmaker uitgevoerd binnen de afdeling toxicogenetica van het LUMC. Het afstudeerverslag behaalde een nominatie voor de Milieuscriptieprijs van de Vereniging Van Milieukundigen (VVM). Het doctoraal examen aan de OUNL werd in

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2004 gehaald en in datzelfde jaar mocht zij van deze universiteit een prijs in ontvangst nemen voor haar inspanningen voor het behoud van de NW-opleiding aan de OUNL, haar bindende rol tussen studenten onderling en tussen studenten en faculteit, haar enorme inbreng in de Facultaire opleidingscommissie en haar uitstekend uitgevoerde afstudeeronderzoek.

Van 2002 tot 2004 zette zij in haar vrije tijd een onderzoek op naar de mogelijkheid om de huidschimmel *Trichophyton rubrum* effectief te behandelen met behulp van een fotodynamische behandeling. In samenwerking met dr. S. Pavel en prof. dr. J.A. Bouwstra schreef zij hier een STW projectvoorstel voor. Dit werd door dr. S. Pavel van de afdeling dermatologie van het LUMC en prof. dr. J.A. Bouwstra van het Leiden/ Amsterdam Center for Drug Research (LACDR) in 2003 ingediend en in 2004 door de technologiestichting STW gehonoreerd. Als wetenschappelijk onderzoeker kreeg zij in 2004 een aanstelling op dit project (STW LKG 6432) bij de afdeling dermatologie van het LUMC. Tevens werd zij gastmedewerker bij het LACDR.

Het onderzoek beschreven in dit proefschrift werd uitgevoerd onder leiding van dr. S. Pavel, prof. dr. J.A. Bouwstra en prof. dr. R. Willemze.

Nawoord

Hoewel veel aspecten in dit onderzoek berusten op een eigen initiatief, is het toch voornamelijk de reactie van de omgeving op deze initiatieven geweest die dit onderzoek hebben doen uitmonden in een proefschrift. De onderzoeksvrijheid die ik bij het LUMC heb gekregen alsmede de samenwerking met PhotoBioChem NV hebben mij de gelegenheid gegeven dit onderzoek te starten, te patenteren en het eerste artikel te publiceren.
Na deze startperiode heeft vooral het LACDR een cruciale, ondersteunende rol gespeeld in dit onderzoek. Vanaf het moment dat ik er met mijn projectvoorstel over een huidschimmel binnenstapte tot op de dag van vandaag. Met de expertise van het LACDR op het gebied van huidpenetratie ging er voor mij een wereld open.
Ook de gastvrijheid binnen de afdeling dermatologie is zeer belangrijk geweest voor dit onderzoek. Kees Tensen zorgde voor mijn “schimmellabje” in gebouw 2 van het LUMC en ik voelde me thuis bij de afdeling huidziekten.
En dan zijn er natuurlijk nog heel veel mensen die allemaal op hun eigen manier één of meerdere steentjes hebben bijgedragen aan dit proefschrift.

Op de eerste plaats zou ik de leden van de gebruikerscommissie van het STW project, STW LKG 6432, willen noemen voor de interesse die zij altijd hebben getoond voor dit onderzoek. Dit geldt in het bijzonder voor Marja Oosterlaken.

Bert Altenburg, ook jij bent heel belangrijk geweest voor mij, omdat je altijd (hoe laat het ook was) klaar stond met advies omtrent patentaanvragen etc.

Binnen het LACDR bij de DDT groep is dat op de eerste plaats Connie geweest. Je zorgde voor een warme ontvangst binnen jullie DDT groep en lette er steeds op dat ik niet vergeten werd. Aan de dagjes uit en kerstfeesten bij DDT houd ik warme herinneringen.

Madeleine Nivard is voor mij een grote steun geweest ten tijde van het schrijven van het projectvoorstel voor dit promotieonderzoek.

Ongelooflijk belangrijk voor mij waren en zijn al vele jaren lang mijn ex-PDT collega’s en vrienden, Laurence, Saskia, Peter en Johan. Ik hoop dat we onze “PDT diners” nog lang kunnen voortzetten.

Mary, jij wast trouw al vanaf 2000 mijn al dan niet beschimmelde glaswerk af. Dat vind ik super!

Mojgan, jij zorgde er bijna 4 jaar lang voor dat er altijd voldoende SC was zodat ik door kon gaan met mijn proefjes. Zelfs toen bij station Schiedam het dak door storm wegwaaide, bleef je onvermoeibaar staan met je emmertje. Een sms'je vertelde me slechts dat je wat later zou komen! Natuurlijk deed je veel meer dan huid verwerken. Je bleek een expert op het gebied van huidpenetratie met teflon diffusiecellen. Maar het allermeest waardeer ik je inzet en goede zorgen.

In de geheimen van de porfyrine chemie werd ik volledig ingewijd door Richard van der Haas en Rob van der Steen. Richard, jij was altijd heel behulpzaam als ik niet uit de porfyrine chemie kwam. Rob, als Sylsens B fabrikant ben je natuurlijk heel belangrijk voor dit onderzoek geweest. Maar het is vooral je enthousiasme geweest voor de porfyrine chemie die zo aanstekelijk werkte dat ook ik me ging verdiepen in de ontwikkeling van nieuwe porfyrine photosensibilisatoren: een nieuw projectvoorstel werd geboren.

Johan Lugtenburg, het was een waar genoegen om gedurende mijn STW LKG4890 projectjaren met je te mogen samenwerken.

Iemand die ik absoluut ook zou willen noemen is Evert Middelbeek van de Open Universiteit Nederland. Jij overleed eind 2004 maar hebt mij altijd heel erg gesteund en me het vertrouwen gegeven dat ook ik best een boekje in elkaar zou kunnen zetten. Je had er dus gewoon bij moeten zijn!

Frans van Nieuwpoort, jij had altijd een helpende hand als er iets met mijn pc aan de hand was. Je begreep ook helemaal dat voor mij het schrijven van een wetenschappelijk artikel minder inspanning vergde dan het elektronisch indienen ervan.

En dan is er de afdeling elektronen microscopie. Hier liep ik ruim drie maanden lang de deur plat en genoot daarbij van de geboden gezelligheid en expertise. Jullie hadden beide in huis! In het bijzonder wil ik graag Henk Koerten bedanken voor de fantastische werkafspraken. Dit leverde een goede samenwerking op en een prachtig artikel tegen een mooie kostprijs. Dan Jos en Joke, jullie waren onmisbaar bij dit stukje onderzoek.

Vooral wanneer er iets niet goed ging, dan stonden jullie altijd meteen klaar en jullie leefden je helemaal in het schimmelgebeuren in!

Aat, je was vele jaren een gezellige kamergenoot maar ook een excellente hulp bij de SEM experimenten. Naast je expertise heb ik je enthousiasme bij dit onderzoek heel erg gewaardeerd!

Hans Rotteveel, op de achtergrond heb jij bij het schrijven van dit proefschrift zeker een rol gespeeld!

Tot slot alle collega's dermatologie en DDT die ik niet genoemd heb: het was een gezellige en leerzame periode!

En dan lieve Joost, wat had ik zonder jou moeten beginnen, jij was er altijd en met steeds heel veel geduld voor míj!

Ester, David, Sven en Karen, jullie zijn fantastisch!