The susceptibility of trichophyton rubrum to photodynamic treatment

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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.

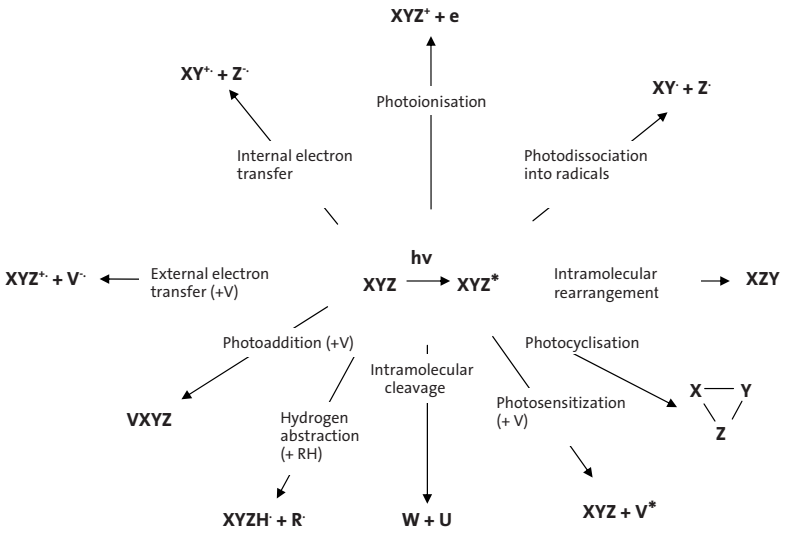

Figure 1.1. Important photochemical reaction types following excitation. The excited state, XYZ* , may be either singlet or triplet.

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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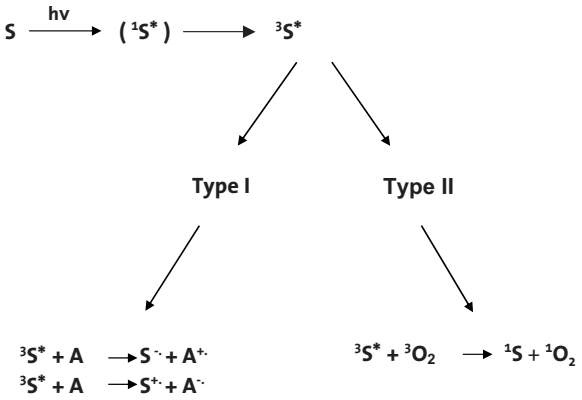

Figure 1.2. Illustration of the photodynamic reaction types that may occur during photodynamic action.

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.

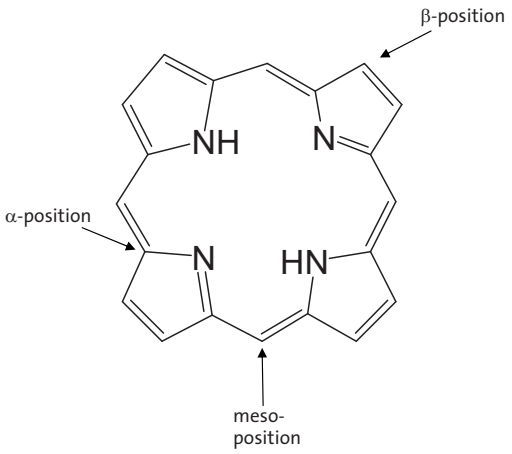

Figure 1.3. Structure of the unsubstituted porphine macrocycle ring.

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.

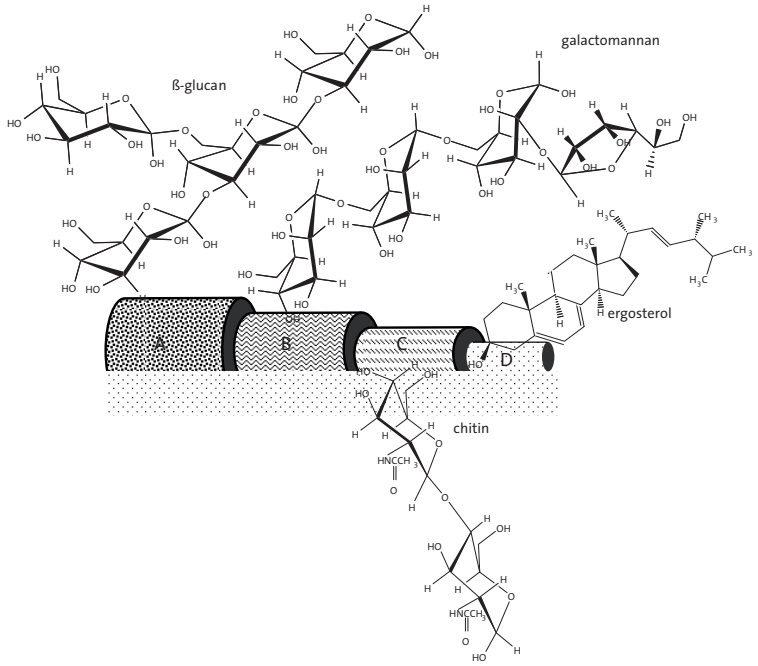

Figure 1.4. Diagrammatic representation of the wall structure of *Trichophyton rubrum*, including the chemical structure of the main units present within the layers that are characteristic for the fungal wall.
(A) Outermost layer of β–1,3-glucans and β-1,6-glucans
(B) Galactomannan, showing α-1,2 en 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.

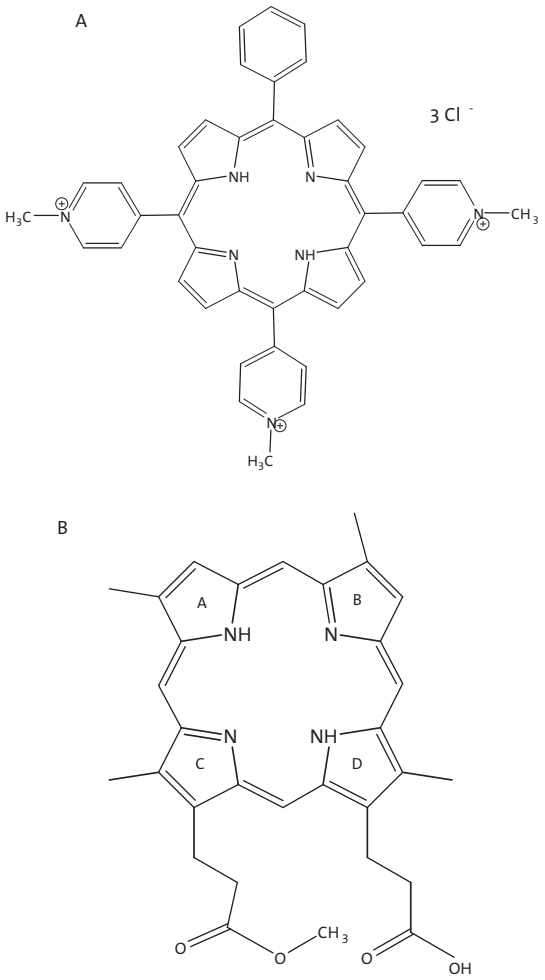

Figure 1.5. Chemical structure of the porphyrin photosensitizer 5,10,15-tris(4-methylpyridinium)-20-phenyl-[21H,23H]-porphine trichloride (Sylsens B, Fig. 1.5A) and deuteroporphyrin monomethylester present as a mixture of 50% with the propyl ester on the D-ring and 50% with this ester on the C-ring (DP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