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Review

A comprehensive overview of photodynamic therapy in the treatment of superficial fungal infections of the skin

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Abstract

Photodynamic therapy (PDT) is a two-step procedure, involving the topical or systemic administration of a photosensitizer followed by selective illumination of the target lesion with visible light, which triggers the oxidative photodamage and subsequent cell death within the target area. In dermatology, PDT has proven to be a useful treatment for a variety of malignant tumors and selected inflammatory diseases. In addition, PDT of several infective viral or bacterial skin diseases has been investigated. These investigations grew out of the positive findings of studies of another important use of PDT: that of disinfection of blood products.

Up to now, little has been published concerning the application of PDT to fungi, probably due to the fact that research funding has been mainly directed towards blood disinfection, and these pathogens show a low risk of transfusion transmission.

However, preliminary findings have demonstrated that dermatophytes and yeasts can be effectively sensitized in vitro by administering photosensitizers belonging to four chemical groups: phenothiazine dyes, porphyrins and phthalocyanines, as well as aminolevulinic acid, which, while not a photosensitizer in itself, is effectively metabolized into protoporphyrin IX. Besides efficacy, PDT has shown other benefits. First, the sensitizers used are highly selective, i.e., fungi were killed at combinations of drug and light doses much lower than that needed for a similar effect on keratinocytes. Second, all investigated photosensitizers lack genotoxic and mutagenic activity. Finally, the hazard of selection of drug resistant fungal strains was never reported.

This paper intends to provide a comprehensive overview of investigative studies about the effects of PDT on yeasts and dermatophytes, and bring attention to this application of PDT which we believe very important in that skin mycosis is so common and PDT is not only cost-effective, but also has the advantages of being highly selective and avoiding the occurrence of drug resistant strains.

Keywords: Photodynamic therapy; Phthalocyanines; Aminolevulinic acid; Phenothiazine dyes; Skin mycosis

1. Introduction

Photodynamic therapy (PDT) combines the topical or systemic administration of a photosensitizer with the selective illumination of the target lesion with visible light, which results in localized oxidative photodamage and subsequent cell death. This technique is now routinely used in dermatology. The FDA has recently approved 5-aminolevulinic acid (ALA) for the photodynamic treatment of actinic keratosis while the European regulatory authorities have approved methyl-aminolevulinate (MAL) for actinic keratosis and basal cell carcinoma. In addition, PDT with these and other sensitizers has been proven, in worldwide laboratory and clinical studies, to be a promising tool for a wide variety of other skin tumors including squamous cell carcinoma, Bowen' s disease and cutaneous T-cell lymphoma, and selected inflammatory and infective

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diseases. This last application grew out of the positive results of research studies concerning photo-induced viral or bacterial inactivation that were conducted with the aim of disinfecting blood products [1,2]. Up to now these blood disinfection studies pay little or no attention to possible applications of PDT to fungi due to the low risk of transfusion transmission of such pathogens [3].

However, superficial skin mycosis, either caused by *Candida* species or dermatophytes, is one of the most frequent diseases in human beings and animals and the search for new therapeutic approaches is stimulated by the fact that standard drug treatments are prolonged and expensive and the appearance of drug resistant strains is more and more frequent in high risk groups such as HIV+ patients and cancer patients undergoing chemotherapy [4]. The present paper aims to provide a comprehensive overview of investigative in vitro and in vivo studies of anti-fungal PDT that have been reported so far.

2. General principles of photosensitization of fungi

Several photosensitizers, mainly belonging to three chemical groups (phenothiazine dyes, porphyrins and phthalocyanines), have been investigated. As a general rule, sensitizers which are medically interesting, are those without dark toxicity, i.e., are devoid of toxicity in the absence of light activation, and, upon irradiation, lack genotoxicity and mutagenicity [5]. The hazard of DNA damages in eukaryotic fungi is furtherly reduced by the presence of a membrane that envelopes the nucleus and may act as a barrier to the penetration of dyes or their high-energy photoproducts [2].

The anti-microbial photodynamic effect has been found strictly dependent on physical and chemical parameters, e.g., absorption peak (λ_{max}), intensity of absorption (ε_{max}) and quantum yield for singlet oxygen.

In addition, the mechanisms and efficiency of cell inactivation are critically dependent on other chemical properties, e.g., the lipophilicity/hydrophilicity balance (log P), the degree of ionization (pK_a), and the presence of electric charged groups that qualify the type and efficacy of cellular mechanisms of uptake of the dye and the pattern of its distribution among the various subcellular compartments [5].

Unlike mammalian cells, fungi are surrounded by a rigid cell wall that is composed principally of soluble and insoluble polysaccharide polymers, like chitin, β-glucans and glycoproteins, and, as a general rule, cellular uptake is negatively influenced by the lipophilicity of the molecules and positively by hydrofilicity and presence of electric charged groups. Following uptake, sensitizers are distributed to subcellular targets and the pattern of localization is very important because targets adjacent to the sensitizer have the greatest probability of being involved in the photoprocess due to the high reactivity and short intrinsic lifetime of the intermediate reactive oxygen species [6]. The biochemical and functional effects of photosensitization include inactivation of enzymes and other proteins and peroxidation of lipids leading to the lysis of cell membranes, lysosomes and mitochondria [7].

The multiplicity of cellular targets in fungi should reduce the risk of selection of photomutant resistant strains and this risk should be furtherly minimized by the lack of mutagenic effects of PDT.

The spectrum of the light used for photoactivation is another critical issue. Light with wavelengths in the far red or near infrared regions can penetrate deeply into mammalian skin. Sensitizers with an absorption peak in these wavebands are needed for the treatment of dermatophytes that can colonize both the stratum corneum and hair follicles. However, photosensitizers absorbing in the blue region may be suitable for the treatment of *Candida* species that can invade only the stratum corneum.

2.1. Phenothiazine dyes

Oxidized phenothiazines have a simple planar, tricyclic skeleton and are normally encountered as cations. The most widely used compounds are methylene blue (MB) and toluidine blue (TBO). Both are efficient producers of singlet oxygen [3] and their maximum wavelength of absorption (λ_{max}) in water is, respectively, 656 nm for MB and 625 nm for TBO [8]. In yeasts, the site of localisation and consequent photodamage, upon light exposure, is the plasma membrane. *Candida albicans* is effectively killed by PDT with either MB or TBO [9] but it is much less susceptible when compared to several prokaryotic bacteria, i.e., *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Propionibacterium acnes* and *Corynebacterium minutissimum* [10]. A possible explanation is that, according to the target theory, the killing effect of photoactivated MB [10] in prokaryotic cells appears to be a single hit process where all the putative targets in the cell are equally susceptible and if damaged can lead to cell death, whereas in the eukaryotes a multihit process is needed where saturation of more than one molecular target is required before cell death occurs. These differences in susceptibility may be amplified by differences in the ratio of cell size to volume. *Candida* species are approximately 25–50 times larger than bacteria and therefore contain a greater number of targets per cell [2].

In the same experimental conditions, the killing rates for fungi were 18–200 fold higher than those determined for keratinocytes suggesting that MB-PDT of fungal infections of the skin may be not only effective but also highly selective [10]. The same drug and light doses did not cause detectable genotoxic and mutagenic effects on both fungi and keratinocytes [11].

Photodynamic inactivation of dermatophytes has been studied as well. Eight strains of dermatophytes (*Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Trichophyton tonsurans*, *Microsporum cookei*, *Microsporum canis*, *Microsporum gypseum*, *Epidermophyton floccosum*, *Nannizia cajetani*) were exposed to UVA irradiation after sensitization with two thiophenes (2,2′:5′,2″-terthienyl and 5-(4-OH-1-butinyl) 2,2′-bithienyl) [13]. A strong and dose-dependent inhibition of the growth of all tested strains was found although a complete inactivation was never obtained [13].

The interest raised by these promising in vitro findings has recently led to a first in vivo study. Oral azole-resistant Candidiasis of SCID beige nude mice, an immunodeficient murine model, was treated with the application of an aqueous solution of MB followed by irradiation with 100 J/cm^2 of laser light with emission peak at 664 nm delivered with a cylindrical diffuser. Effects were dependent on light and drug doses [12]. The authors concluded that MB-PDT might represent an effective, non-toxic, simple, inexpensive and repeatable therapy of oral Candidiasis in immunodeficient HIV+ patients [12] which, as it is well known, often select resistant fungal strains. However, the therapeutical technique that they used has the serious limitation of not covering the concomitant esophageal infection which is noticed very often concomitantly in such patients. Another additional drawback of this study is that results of follow-up examinations were not reported.

2.2. Porphyrins

Hematoporphyrin derivative (HpD) is a complex mixture of mono- and oligo-meric porphyrins (Pp) derived from blood. It has various absorption peaks in the 400–650 nm region and its anti-fungal properties have been investigated since the early 1980s [14].

Within a broad range of concentrations, HpD is not toxic for cells in dark conditions [4,5] but, after irradation it can effectively kill fungal cells although mechanisms of photosensitization are quite different from those described for MB and other phenothiazine dyes. HpD is not uptaken by *Saccharomyces cerevisiae* and *C. albicans* [14,15] and the photocytotoxic activity is mainly promoted by unbound dye molecules in the bulk aqueous medium. These, after irradiation, cause an initial limited alteration of the cytoplasmic membrane that allows for the penetration of the dye into the cell, the translocation to the inner membranes and the consequent photodamage of intracellular targets [7]. At a biochemical level, the photoprocess involves mainly the peroxidation of the lipids and, only to a minor extent, the inactivation of proteins of the cell wall [7].

In order to determine the importance of sterol oxidation, sterol auxotrophic strains of *S. cerevisiae* were grown on media containing sterols with different levels of unsaturation before photosensitization [16]. Cells grown on a completely saturated sterol, cholestanol, were markedly more resistant to the photosensitising process and photodegradation of the native unsaturated yeast sterol, ergosterol, caused the substantial loss of cell viability.

Recently, several new Pp derived compounds have been evaluated for their possible anti-fungal activity. These drugs have been synthesized in order to obtain chemically pure dyes with an intense absorption peak in the 650–700 nm waveband, and high quantum yields for singlet oxygen. Polarity of the molecule is a critical feature. Neutral amphiphilic meso-arylglycosylporphyrins [17] and water-soluble diamino-acid derivatives of Pp [18] are characterized by a greater uptake and, consequently, a greater activity against yeasts in comparison to hydrophobic molecule such as HpD, chlorines and benzoporphyrin derivatives.

Photo-activated hydrophilic porphyrinic compounds (deuteroporphyrin, deuteroporphyrin monomethylesther and 5,10,15-tris(4-methylpyridinium)-20-phenyl-(21H, 23H)-porphine trichloride) showed a great activity against *T. rubrum* as well. Again, their activity was found greater than that of lipophilic phthalocyanines (ZnPc, PcS_4 and $AlPcS_4$) and Photofrin® that displayed nothing more than a fungistatic effect for about 1 week [19].

The biological role of photodynamic reactions mediated by naturally occurring endogenous porphyrins is presently being debated. Irradiation with 20–50 J/cm^2 of broadband visible light without the addition of exogenous sensitizers was found to produce oxygen-dependent lethal effects of plasma membranes and mitochondria of *Candida guilliermondii* [20]. However in another study [9], the viability of suspensions of *C. albicans* was not affected by irradiation with 66 J/cm^2 of red laser light (632.8 nm).

2.3. 5-Aminolevulinic acid

5-Aminolevulinic acid (ALA) is not a photosensitizer in itself but all eukaryotic cells metabolize it into a very active endogenous sensitizer, protoporphyrin IX (PpIX), in the enzymatic pathway to heme [21]. However, in comparison to mammalian cells, fungi display some differences in the enzymatic machinery: coproporphyrinogen oxidase is found in the cytoplasm and both ALA-synthase and ALA-dehydratase are the rate limiting steps and their activity is controlled by the intracellular free heme pool [22]. Therefore the content of PPIX may be increased by the addition of iron chelators, e.g., EDTA, hydroxypyridinone and 2,2′-dipyridil, that may inhibit the conversion of PpIX itself into heme [23].

PpIX may be activated by wavelengths ranging from UVA to the visible wavebands with a maximum peak in the Soret band at 375–405 nm and a lower peak at 630–633 nm. Upon exposure to light, PpIX induces cytotoxic

effects through oxygen-dependent photochemical reactions that damage mainly the mitochondria, where PpIX is synthesized, and plasma membranes [23].

Prolonged irradiation causes the additional alteration of other cytoplasmic structures and the inhibition of the synthesis of DNA and RNA [24], but genotoxic and mutagenic effects were never detected in yeasts [22].

The clinical efficacy of ALA-PDT in the treatment of fungal infections of human skin has been recently investigated with an open pilot study enrolling nine patients with interdigital mycosis of the feet. Before therapy, skin scrapings of lesional skin were inoculated on Sabouraud's dextrose agar containing antibacterial antibiotics. Colonies of *C. albicans* grew in three cases, *Trichophyton* dermagrophytes in four and *T. rubrum* in two. All colonies showed a strong red fluorescence after incubation with 20% ALA water solution and irradiation with Wood's lamp (Fluolight, Saalman GmbH, Germany) (Fig. 1). The treament protocol provided for the application of a 20% ALA cream under an occlusive dressing, followed, 4 h later, by the irradiation of 75 J/cm^2 of broadband red light. The first follow-up visit took place after 7 days and no further treatment was delivered if lesions were not clinically evident and direct microscopic examination proved negative. Otherwise, three additional weekly treatments were delivered. Four weeks after the last treatment patients had a final follow-up clinical and laboratory examination.

Clinical and microbiological recovery was seen in six out of nine patients after one (four cases) or four (two cases) treatments. However, only two patients (one with *C. albicans* and one with *T. mentagrophytes*) had a persistent remission at the final visit. Overall tolerability was good in all patients.

It was unclear why the apparently good therapeutic effect was followed by quick recurrence in almost all patients. A first possible explanation is that "in vivo" environmental conditions, i.e., temperature, humidity and pH of the interdigital skin, could induce a poor cell uptake of ALA and a deficient biosynthesis of PpIX. In addition the non-uniform delivery of light and/or ALA cream due to the irregular tridimensional shape of this peculiar anatomical area must be taken into account [25].

Fig. 1. A strong red fluorescence is detected after incubation of colonies of *Candida albicans* with 20% ALA water solution and irradiation with Wood's lamp.

2.4. Phthalocyanines

A high number of phthalocyanines (Pc) are currently available. All these compounds have in common a Pp-like chemical structure characterized by the condensation of benzene rings with pyrrole moieties and are characterized by high singlet oxygen quantum yields and high extinction coefficient in the far red (680–720 nm) spectral region [5]. However, they differ from each other in chemical properties that may be tuned by modifying the central metal/semi-metal atom and the number and type of side chains. The central metal ligand plays a crucial role in the photobiological activity influencing the triplet yield and the triplet lifetime of the compound and the introduction of polar substituents on the side chains which modify the overall balance of polarity.

The hydrophilicity/hydrophobicity balance is a very important chemical property when considering Pc uptake and its localization into micro-organisms. The phthalocyanine macrocycle is essentially hydrophobic but hydrophobic compounds were found poorly effective [26]. However, also hydrophilic mono- and tetra-sulfonated AlPc were not taken up efficiently and did not inactivate the yeast *Kluyveromyces marxianus* [26] whereas an appreciable amount of another water-soluble compound, the mono-sulfonated ZnPcS, was tightly bound to intracellular loci and showed a high photosensitizing activity [5] in *S. cerevisiae*.

This discrepancy may be explained by recent findings showing that the presence of cationic charges is necessary for inactivation of *C. albicans*, but an excess of charges, especially if homogeneously distributed, causes a decrease in activity [29]. On the basis of these findings, a tetracationic ZnPc bearing four aminoalkilated peripheral substituents (called RPL068) has been recently developed which is highly capable of inactivating even strains of *C. albicans* which are multi-drug resistant. Additional advantages of Pc-PDT consist in the lack of selection of PDT resistant strains even after multiple treatments and the much greater photosensitizing activity in *C. albicans* than in mouse and human keratinocytes and fibroblasts. In a mouse model, a single treatment with RPL068 and red light proved very effective and selective in the treatment of *Candida* infection of the skin [30].

Some authors have investigated the incorporation of Pc in liposomes as an alternative strategy for enhancing cell uptake of yeasts [5]. However, results were

disappointing because the size of liposomes ranges from 20 to 100 nm [27] and only globular structures with diameter of less than 5.8 nm can pass through the cell membrane of yeasts [28].

Mutagenic effects of photodynamic treatment with ClAl-Pc and RPL068 were not found in *K. marxianus* [24] and *C. albicans* [30].

3. Conclusions and perspectives

Results of experimental investigations have demonstrated that dermatophytes and yeasts can be effectively sensitized in vitro by several dyes belonging to three chemical groups: phenotiazinum, Pp and Pc. In addition, they can effectively metabolize ALA to PpIX. Besides being effective, antifungal PDT is selective because fungi can be killed at dose rates much lower than that which kills keratinocytes. Absence of genotoxic and mutagenic effects on both fungal cells and keratinocytes seems a fundamental premise for the long-term safety of the treatment, e.g., the lack of selection of drug-resistant fungal strains and cancerogenesis.

In spite of the growing number of promising findings on cell cultures, only recently a small number of papers has reported findings of in vivo photosensitization of mycoses of laboratory animals and humans.

However, it is worthwhile to emphasize that the most recent experimental in vitro [13,17–19] and in vivo [29] studies have been conducted with sensitizers that have been synthesized specifically for antimycotic PDT. This may represent a signal of a new interest on the part of drug companies and university laboratories.

Hopefully this interest may also help in attracting funding for those further studies that are needed in order to assess if PDT may represent a new possible treatment approach for skin mycosis.

4. Abbreviations

PDT	photodynamic therapy
ALA	5-aminolevulinic acid
MAL	methyl-aminolevulinate
MB	methylene blue
TBO	toluidine blue
HpD	hematoporphyrin derivative
Pp	porphyrin
PpIX	protoporphyrin IX
Pc	phthalocyanine

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