Microbiological Research **163** (2008) 1—12

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Microbiological Research

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REVIEW

Antifungal photodynamic therapy

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Received 12 June 2007; received in revised form 2 August 2007; accepted 5 August 2007

KEYWORDS
Photodynamic antimicrobial chemotherapy;
Yeasts;
Dermatomycetes;
Photosensitisers

Summary

In photodynamic antimicrobial chemotherapy (PACT), a combination of a sensitising drug and visible light causes selective destruction of microbial cells. The ability of light–drug combinations to kill microorganisms has been known for over 100 years. However, it is only recently with the beginning of the search for alternative treatments for antibiotic-resistant pathogens that the phenomenon has been investigated in detail. Numerous studies have shown PACT to be highly effective in the *in vitro* destruction of viruses and protozoa, as well as Gram-positive and Gram-negative bacteria and fungi. Results of experimental investigations have demonstrated conclusively that both dermatomycetes and yeasts can be effectively killed by photodynamic action employing phenothiazinium, porphyrin and phthalocyanine photosensitisers. Importantly, considerable selectivity for fungi over human cells has been demonstrated, no reports of fungal resistance exist and the treatment is not associated with genotoxic or mutagenic effects to fungi or human cells. In spite of the success of cell culture investigations, only a very small number of *in vivo* animal and human trials have been published. The present paper reviews the studies published to date on antifungal applications of PACT and aims to raise awareness of this area of research, which has the potential to make a significant impact in future treatment of fungal infections.

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doi:10.1016/j.micres.2007.08.001

Introduction

The incidence of superficial and deep-seated fungal infections has increased markedly over the last 20 years. Several reasons have been proposed for the rise in incidence of fungal infections, including the increasing use of antineoplastic and immunosuppressive drugs, broad-spectrum antibiotics, prosthetic devices and grafts, and more aggressive surgery. Patients with burns, neutropenia, HIV infection and pancreatitis are also predisposed to fungal infection (Eggimann et al., 2003). Fungi have become increasingly recognised as major pathogens in critically ill patients. In fact, *Candida* spp. are currently the third leading cause of bloodstream infections in the USA and disseminated candidiasis is associated with a mortality in excess of 25% (Kibbler et al., 2003). Furthermore, *Aspergillus* spp. are the leading cause of pneumonic mortality in acute leukaemia and bone marrow/haematopoietic stem cell transplant recipients and have surpassed cytomegalovirus infection in this setting (Walsh et al., 2000).

In comparison to antibacterial therapy, antifungal treatment is limited to a very small number of drug substances. Very often, treatment is prolonged and serious side effects and drug–drug interactions are common (Katz, 1997; Hay, 1999). In many cases, treatment is also ineffective (Cohen and Scher, 1994; Walsh et al., 2000). In addition, the incidence of resistance to antifungal agents may be increasing (Denning, 1995; Johnson et al., 1995; Walsh et al., 2000; Dodgson et al., 2004; Pujol et al., 2004; Blignaut et al., 2005) with drug-resistant fungal strains particularly common causative pathogens of infection in high-risk patient groups, such as HIV/AIDS patients (Johnson et al., 1995; Pankhurst, 2001). Accordingly, alternative antifungal strategies are being actively sought.

In photodynamic antimicrobial chemotherapy (PACT), a combination of a sensitising drug and visible light causes selective destruction of microbial cells. The ability of light–drug combinations to kill microorganisms has been known for over 100 years (Moan and Peng, 2003). However, it is only recently with the beginning of the search for alternative treatments for antibiotic-resistant pathogens that the phenomenon has been investigated in detail. Numerous studies have shown PACT to be highly effective in the *in vitro* destruction of viruses and protozoa, as well as Gram-positive and Gram-negative bacteria and fungi. The present paper reviews the research published to date on antifungal applications of PACT.

Photodynamic therapy

Mechanism of action

The detailed mechanism of action of PACT has been discussed extensively elsewhere (Dougherty et al., 1998; Kalka et al., 2000; Konan et al., 2002). Briefly, it results from the interaction of photons of visible light, of appropriate wavelength, with intracellular concentrations of photosensitising molecules known as photosensitisers. Photosensitisers have a stable electronic configuration, which is in a singlet state in their lowest or ground energy level, $^{\circ}$PS (Konan et al., 2002). This means that there are no unpaired electron spins (Isaacs, 1992; Kalyanasundaram, 1992). Following absorption of a photon of light of specific wavelength (Figure 1), a molecule is promoted to an excited state, $^{\circ}$PS*, which is also a singlet state and is short lived with a half life between 10^{-6} and 10^{-9} s (Dougherty et al., 1998; Konan et al., 2002). The photosensitiser can return to the ground state by emitting a photon as light energy, or, in other words, by fluorescence, or by internal conversion with energy lost as heat. Alternatively, the molecule may convert to the triplet state, ^{2}PS*. This conversion occurs via intersystem crossing which involves a change in the spin of an electron (Oschner, 1997). The triplet state photosensitiser has lower energy than the singlet state but has a longer lifetime.

The singlet state sensitiser can interact with surrounding molecules via Type I reactions, while the triplet state sensitiser can interact with its

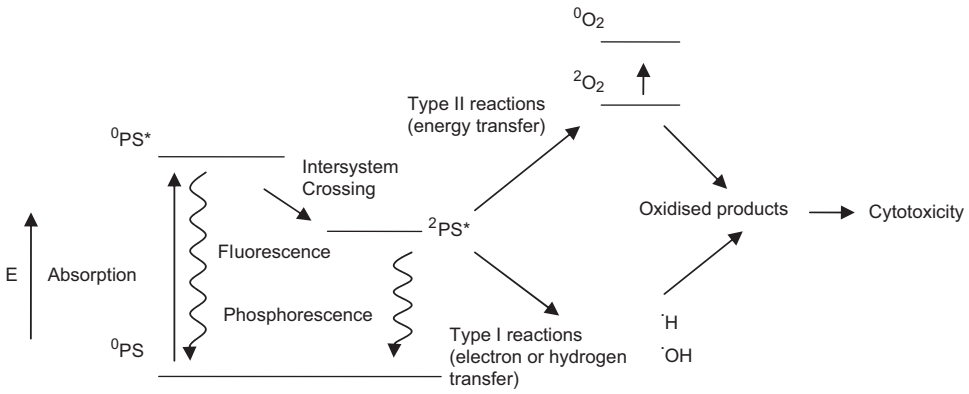

Figure 1. The mechanism of action of photodynamic therapy. Numbers in superscripts denote the number of unpaired electron spins in each molecule. Adapted from Konan et al. (2002).

surroundings via Type II reactions. The former type of reaction leads to the production of free radicals or radical ions, via hydrogen or electron transfer. These reactive species, after interaction with oxygen, can produce highly reactive oxygen species, such as the superoxide and peroxide anions, which then attack cellular targets (Kalka et al., 2000). However, Type I reactions do not necessarily require oxygen and can cause cellular damage directly, through the action of free radicals, which may include sensitiser radicals. Type II reactions, by contrast, require an energy transfer mechanism from the triplet-state sensitiser to molecular oxygen, which itself normally occupies the triplet ground state, $^2O_2$ (De Rosa and Bentley, 2000). Although possessing a short lifetime of approximately 10^{-6} s, a sufficient concentration of highly cytotoxic singlet oxygen, $^{\circ}O_2$, is produced to induce irreversible cell damage (Dougherty et al., 1998; Kalka et al., 2000). In addition, the photosensitiser is not necessarily destroyed, but can return to its ground state by phosphorescence without chemical alteration and may be able to repeat the process of energy transfer many times (Oschner, 1997). Alternatively, the sensitiser may return to ground by transferring its energy to molecular oxygen, and may even be destroyed by photobleaching due to oxidation (Moan et al., 1997). Evidently, many effects of PACT are oxygen-dependent and rely on the oxygen tension within the target. Type I and Type II reactions can occur simultaneously and the ratios between the two depend on the photosensitiser, substrate, oxygen concentration and sensitiser to substrate binding (Kalka et al., 2000). Singlet oxygen is, however, widely believed to be the major damaging species in PACT (Peng et al., 1997; Gannon and Brown, 1999; Konan et al., 2002). Due to its extreme reactivity, singlet oxygen has a short lifespan in a cellular environment and limited diffusivity, allowing it to travel only approximately 0.1 μm (Moan, 1990).

Photosensitisers

The efficacy of certain types of dye against microbial species formed the basis of modern chemotherapy over 100 years ago. The selectivity, particularly of cationic dyes, for bacteria over mammalian cells was used by Ehrlich and Browning to develop early synthetic antibacterials. However, much of the impetus for this work was lost at the inception of the antibiotic era, when the action of penicillin was seen as miraculous. The recent renaissance in the use of dyes and their derivatives in cancer treatment (photodynamic therapy, PDT) relies on the fact that the dyes act as photosensitisers.

In order for a molecule to act as an efficient photosensitiser, it must possess the ability to absorb visible light, becoming excited to the triplet state, and then transfer its energy economically to molecular oxygen. Molecules possessing such characteristics are typically rigid planar structures possessing a high degree of conjugation. The major photosensitiser classes employed to date in PACT include the porphyrins, the phthalocyanines and the phenothiaziniums (Figure 2). The phenothiaziniums have simple tricyclic planar structures, typically cationic in nature. The most widely used compounds are methylene blue (MB) and toluidine blue (TBO). Both are efficient producers of singlet oxygen and the maximum absorption wavelength in water is 656 nm for MB and 625 nm for TBO, respectively. The porphyrins are heterocyclic macrocycles derived from four pyrrole-like subunits interconnected via their α carbon atoms via methine bridges. The absorption spectrum of

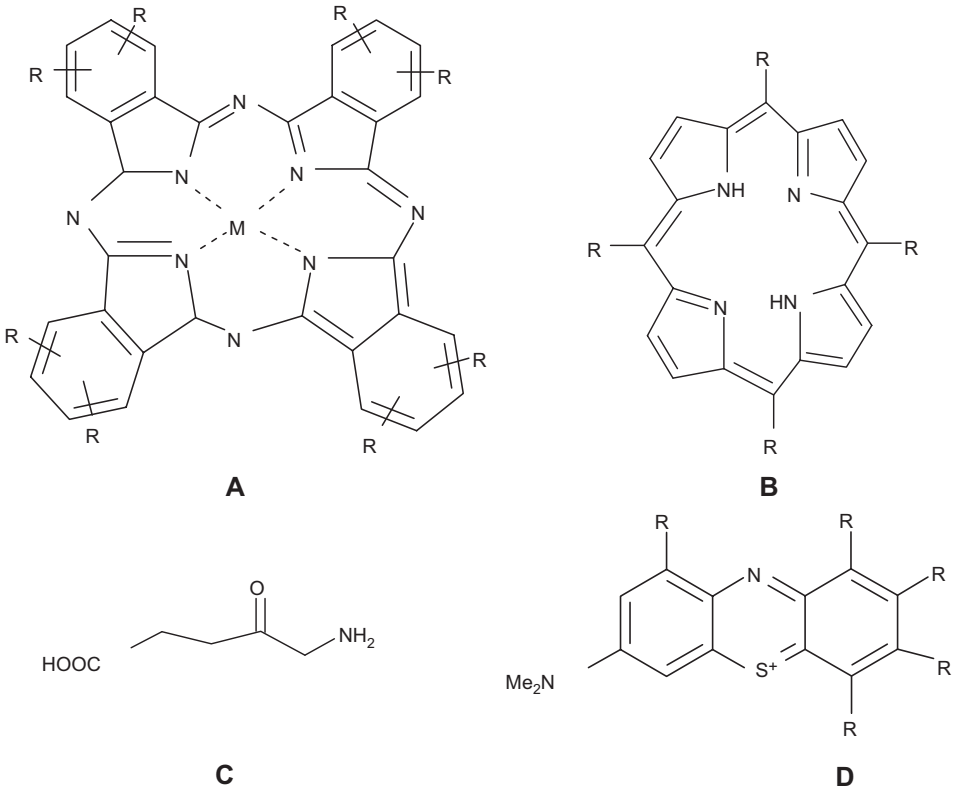

Figure 2. Basic chemical structures of phthalocyanine (A), porphyrin (B), 5-aminolevulinic acid (C) and phenothiazinium (D) photosensitisers used in antifungal photodynamic antimicrobial chemotherapy.

porphyrins exhibits a maximum in the Soret band in the visible region of the electromagnetic spectrum between 360 and 400 nm, followed by 4 smaller peaks between 500 and 635 nm (Q-bands) (Kalka et al., 2000). The pyrrole groups in phthalocyanines are conjugated to benzene rings and bridges by aza nitrogens rather than methane carbons. This causes the absorption spectrum to shift to longer wavelengths and the Q bands to become more intense than the Soret peak (Bonnett et al., 2001).

Localisation of photosensitisers and selectivity for microorganisms

It is now well known that cationic photosensitisers are more efficient than their neutral or anionic counterparts in the photodynamic killing of microbial cells. Cationic photosensitisers are more effective, especially as broad-spectrum antibacterials, than their anionic counterparts (Wainwright, 1998), as shown by their greater activity against Gram-negative bacteria, which have a more complex structure due to the presence of an outer membrane. The cell envelope of Gram-negative bacteria consists of an inner cytoplasmic membrane and an outer membrane that are separated by the peptidoglycan-containing periplasm. The outer membrane, which is highly negatively charged, forms a physical and functional barrier between the cell and its environment (Nikaido, 1990). It has been shown that anionic and neutral photosensitisers can become effective against Gram-negative bacteria when co-administered with a cationic agent such as polymyxin (Malik et al., 1992). However, for simplicity and because even against more susceptible Gram-positive bacteria, cationic photosensitisers appear to be more effective (Wainwright, 1998; Jori and Brown, 2004; Wainwright and Crossley, 2004), these cationic agents are the predominant type used in PACT.

To date, there have been several reports on the use of photosensitisers and light to kill both yeasts and other fungi. However, there has been much less systematic study on the types of physicochemical properties necessary in a photosensitiser in order to make it effective in mediating photodynamic killing of such microorganisms. Fungi present much more complex targets than bacteria. For example, yeasts, which constitute a large group of rather disparate eukaryotic organisms, are enveloped by a thick external wall composed of a mixture of

glucan, mannan, chitin and lipoproteins and separated from the plasma membrane by a periplasmic space. However, the available evidence suggests that the response of such cells to photodynamic processes is less strictly controlled by structural factors as compared with bacteria (Paardekopper et al., 1995). Nevertheless, similarities with mammalian cells should be considered and this may indicate the use of cationic photosensitisers, rather than their anionic counterparts, since the latter exhibit facile uptake by mammalian cells (Bonnet, 1995).

Uptake of exogenous substances by fungi is generally adversely affected by lipophilicity and positively affected by hydrophilicity and the presence of charged groups. Following uptake, photosensitisers are distributed to subcellular targets. The pattern of localisation is important, as targets adjacent to the photosensitiser have the greatest probability of being involved in photodynamic processes, due to the high reactivity and short lifetime of the singlet oxygen generated. The biochemical and functional effects of photosensitisation include inactivation of enzymes and other proteins and peroxidation of lipids, leading to the lysis of cell membranes, lysosomes and mitochondria (Bertoloni et al., 1987). Thus, singlet oxygen generated by excitation of photosensitisers is a non-specific oxidising agent. Consequently, there is no cellular defence against it. Indeed, antioxidant enzymes such as catalase and superoxide dismutase are inactivated by it. This means that there should be no difference in susceptibility to PACT between organisms resistant to conventional antifungals and their naïve counterparts. The high reactivity of singlet oxygen has other advantages, because even though the localisation of the photosensitiser may be determined by its physiochemical properties, the diffusion of singlet oxygen should be sufficient to be able to inactivate other structures and biomolecules. Therefore, it is unlikely that fungi could readily evolve resistance to singlet oxygen. In addition, photodynamic processes have never been associated with mutagenic effects in microorganisms. Moreover, singlet oxygen is only present during illumination and fungi are not continuously exposed to it, as they are with conventional antifungals. Furthermore, singlet oxygen cannot travel to other sites in the body, such as the intestinal tract, during treatment. These latter facts make development of resistance even more unlikely.

It has been widely noted that *Candida albicans*, like other yeasts is slightly more difficult to kill by PACT than Gram-positive bacterial cells, necessitating higher drug and light doses (Zeina et al., 2002). This has been attributed to the presence of a nuclear membrane in the yeasts, the greater cell size and the reduced number of targets for singlet oxygen per unit volume of cell (Zeina et al., 2001; Codling et al., 2003; Demidova and Hamblin, 2005). However, it has been shown that the photosensitiser and light doses producing high levels of kill in yeasts *in vitro* do not kill appreciable numbers of human cells under the same conditions and cause no detectable genotoxic or mutagenic effects (Zeina et al., 2003). Should photodynamic killing of fungi be carried out *in vivo*, then the limited diffusion distance of singlet oxygen from its site of generation and the fact that illumination would be limited to the area of infection means that selectivity for fungi over host cells would be further enhanced.

In vitro studies

As antifungal PACT is very much a developing science, the vast majority of published work has understandably centred on *in vitro* laboratory investigations. Various fungi, photosensitisers and irradiation protocols have been employed. In most cases, complete kill of both yeasts and dermatomycetes have been readily achieved. Critically, no reports on development of resistance to antifungal PACT currently exist and the treatment has not been associated with mutagenic effects or genotoxicity in either fungi or cultured human cells. The most extensively investigated photosensitiser classes investigated in *in vitro* antifungal PACT studies have been the phenothiaziniums, the porphyrins, both exogenously delivered and endogenously generated, and the phthalocyanines.

Phenothiaziniums

The phenothiaziniums, such as TBO and MB are known to localise in the plasma membrane of yeasts. Consequently, this is the cellular structure damaged upon illumination and it has been proposed that the increased permeability resulting from such damage is the reason for cell death (Paardekopper et al., 1992). *Candida* species are effectively killed by PACT, whether grown planktonically (Wilson and Mia, 1993; De Souza et al., 2006) or in biofilm (Donnelly et al., 2007). However, they are considerably less susceptible to photodynamic killing than a number of prokaryotic bacteria, including *Staphylococcus aureus* and *Propionibacterium acnes* (Zeina et al., 2002). In fact, doses of TBO as high as 2.0 mg ml^{-1} have been required to induce high levels ($>99\%$) of kill in planktonic *C. albicans* upon illumination. Similarly,

C. albicans biofilms required TBO doses of 5.0 mg ml^{-1} to achieve high levels of kill (Donnelly et al., 2007). High doses (200 J cm^{-2}) of red light (635 nm) have also been required in the photodynamic killing of *C. albicans* (Donnelly et al., 2007).

It has been proposed (Zeina et al., 2002) that, according to the target theory, the killing effects of photoactivated phenothiazines in prokaryotic cells appears to be a single hit process. In such a process, all putative targets in the cell are equally susceptible and if damaged can lead to cell death. Conversely, in eukaryotes, a multi-hit process is required, whereby 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 cell volume. *Candida* species, for example, are approximately 25–50 times larger than bacteria and, therefore, contain a greater number of targets per cell (Zeina et al., 2001; Demidova and Hamblin, 2005). Importantly, however, no evidence currently exists to suggest the ability of yeast cells to develop resistance to phenothiazinium-mediated photodynamic killing.

It is interesting to note that, under the same experimental conditions, with MB as the photosensitiser, the killing rates for *C. albicans* were 18–200 times higher than those determined for keratinocytes (Zeina et al., 2002). Moreover, no genotoxic or mutagenic effects were observed (Zeina et al., 2003). This suggests that despite requiring higher drug and light doses than photodynamic eradication of prokaryotes, PACT mediated by the phenothiaziniums may be both effective and selective in the treatment of *Candida* skin infections.

One study investigated photodynamic destruction of dermatomycetes (*Trichophyton interdigitale*, *Trichophyton rubrum*, *Trichophyton tonsurans*, *Microsporum cookei*, *Microsporum canis*, *Microsporum gypseum*, *Epidermophyton floccosum*, *Nannizia cajetani*) using two thiophene photosensitisers (2,2′:5′,2″-terthienyl and 5-(4-OH-1-butinyl) 2,2′-bithienyl) (Romagnoli et al., 1998). A strong and dose-dependent inhibition of growth of all tested strains was observed. However, complete inactivation was never obtained and the UVA irradiation employed is undesirable for clinical use.

Porphyrins

The anti-fungal properties of porphyrins have been investigated extensively since the early 1980s (Ito, 1981). Upon irradiation, these molecules can effectively kill yeast cells. However, the mechanism of photodynamic action is quite different from that of the phenothiaziniums. The porphyrins are not taken up by yeast cells and the phototoxic activity is mainly due to the photoactivation of unbound photosensitiser molecules in the bulk aqueous medium (Ito, 1981; Bertoloni et al., 1993; Bliss et al., 2004). Following irradiation, the porphyrins cause an initial limited alteration of the cytoplasmic membrane. This then allows penetration of the photosensitiser into the cell, enabling translocation to the inner membranes and, upon continued irradiation, photodynamic damage of intracellular targets (Bertoloni et al., 1987). At a biochemical level, the photoprocess involves mainly lipid peroxidation, photodegradation of unsaturated sterols and, to a minor extent, inactivation of cell wall proteins (Bertoloni et al., 1987). The hydrophilicty of porphyrins is critical to their ability to act as efficient photosensitisers of yeast cells, with highly water soluble porphyrins much more effective in photodynamic killing of such cells than their more hydrophobic counterparts (Zoladek et al., 1997; Carré et al., 1999). Unlike phenothiaziniums, such as TBO, porphyrins do not exhibit significant dark toxicity to yeast cells (Donnelly et al., 2007).

The role of photodynamic reactions mediated by naturally occurring endogenous porphyrins has been the subject of much recent debate. Irradiation (20–50 J cm^{-2}) of broadband white light without the addition of exogenous photosensitisers caused oxygen-dependent damage to plasma membranes and mitochondria of *Candida guilliermondii* (Fraikin et al., 1996). However, the viability of *C. albicans* suspensions was not significantly affected by irradiation (66 J cm^{-2}) of red light (632.8 nm) (Wilson and Mia, 1993).

Studies designed to mimic *in vivo* conditions have demonstrated that the efficiency of photodynamic killing of *C. albicans* by porphyrins is reduced by the presence of serum and albumin in concentrations that might be found in a wound (Chabrier-Rosélló et al., 2005; Lambrechts et al., 2005a). In addition, it has also been suggested that the doses of cationic porphyrins and light required to induce high levels of *C. albicans* kill are likely to result in substantial fibroblast damage. In contrast, the photodynamic doses required to kill *S. aureus* were significantly lower (Lambrechts et al., 2005b). However, in contrast to an adaptive response by *C. albicans* germ tubes to oxidative stress mediated by hydrogen peroxide, there was no adaptive response to singlet-oxygen mediated stress by photodynamic action (Chabrier-Rosélló et al., 2005). In addition, several of the virulence mechanisms, such as biofilm formation, used by microorganisms to

attenuate both the host response and the effects of antimicrobial chemotherapy, are apparently not fully effective during porphyrin-mediated photodynamic killing of *C. albicans* (Chabrier-Rosélló et al., 2005). Moreover, it is notable that porphyrin-mediated photodynamic killing does not induce mutagenicity in yeasts (Zoladek et al., 1997) and there is no evidence to support the ability of yeast cells to develop resistance to porphyrin-mediated photokilling.

Upon irradiation with broadband white light, several hydrophilic porphyrinic photosensitisers (deuteroporphyrin, deuteroporphyrin monomethylester and 5,10,15-tris(4-methylpyridinium)-20-phenyl-(21 H, 23 H)-porphine trichloride (Sylsens B) have been shown to exhibit toxic effects towards the dermatomycete *T. rubrum* in suspension culture, as well as its isolated microconidia (Smijs and Schuitmaker, 2003). A follow-up study using quinolino-(4,5,6,7-efg)-7-de-methyl-8-deethylmesoporphyin dimethylester yielded only a fungistatic effect. However, red light irradiation of Sylsens B produced complete inactivation of fungal spores and destruction of fungal hyphae (Smijs et al., 2004).

5-aminolevulinic acid

Although not a photosensitiser itself, 5-aminolevulinic acid (ALA) is a naturally occurring precursor in the biosynthetic pathway of haem (Brouillet et al., 1975). However, administration of excess exogenous ALA leads to accumulation of the potent photosensitiser protoporphyrin IX (PpIX). ALA is the most commonly used agent in photodynamic therapy of superficial cutaneous neoplasias, where reduced ferrochelatase and enhanced porphobilinogen deaminase activity and a disordered *stratum corneum* barrier lead to selective PpIX accumulation in such lesions. 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 haem pool (Moretti et al., 2000). Therefore, the content of PpIX may be increased by the co-delivery of iron chelators, such as EDTA, hydroxypyridone and 2,2′-dipyridil, that may inhibit the conversion of PpIX to haem (Strakhovskaya et al., 1998).

Upon exposure to light, PpIX induces cytotoxic effects through photochemical reactions that damage the plasma membranes and the mitochondria where PpIX is synthesised (Strakhovskaya et al., 1998). Prolonged irradiation causes the additional alteration of other cytoplasmic structures and the inhibition of the synthesis of DNA and RNA (Paardekopper et al., 1994), but genotoxic and mutagenic effects have never been detected in yeasts (Moretti et al., 2000). The ability of ALA-induced PpIX to kill *C. albicans* upon irradiation has been demonstrated *in vitro* (Monfrecola et al., 2004; Donnelly et al., 2005). It was found that prolonged incubation with ALA was also toxic to *C. albicans* in the absence of irradiation (Donnelly et al., 2005).

Only two studies to date have investigated the potential of ALA-induced PpIX to kill dermatomycetes. *T. rubrum* was shown to synthesise PpIX from exogenously supplied ALA (Kamp et al., 2005). However, 10–14 days' incubation with 1–10 mM ALA were required to induce maximal PpIX concentrations. Increasing the ALA concentration led to reduced growth due to a concomitant decrease in suspension pH. Only incubation with 100 mM ALA for 2 h followed by irradiation (635 nm, 100 J cm^{-2}) caused a significant reduction in viability of *T. interdigitale* microconidia (Donnelly et al., 2005). Incubation with lower ALA concentrations for 2 h and incubation for 30 min produced no such significant reductions. However, significant kill rates were achieved, with even moderate ALA concentrations when the microorganism was incubated for 6 h followed by irradiation. ALA, in the absence of irradiation produced no significant kill of *T. interdigitale*. These latter points, perhaps, reflect the slower metabolic rate and innate robustness of dermatomycete microconidia compared with yeast cells.

It should be noted that, if the administered dose is sufficiently high, ALA has the potential to induce photosensitising concentrations of PpIX in normal human tissue by saturating the normal haem biosynthetic pathway. As a result, ALA is unlikely to be particularly useful in clinical treatment of superficial fungal infections of the skin, nails or mucous membranes.

Phthalocyanines

The phthalocyanine macrocycle is essentially hydrophobic and hydrophobic photosensitisers are known to be less effective in PACT (Berg et al., 1989). In addition, hydrophilic mono- and tetrasulphonated aluminium phthalocyanines were not taken up efficiently and did not inactivate the yeast *Kluyveromyces marxianus* (Berg et al., 1989). However, an appreciable amount of water-soluble monosulphonated zinc phthalocyanine was tightly bound to intracellular loci and showed a high photosensitising activity in *S. cerevisiae* (Bertoloni et al., 1992). It has been proposed that cationic water-soluble phthalocyanines may be more reliably taken up by

yeast cells, but that such photosensitisers should not contain an "excessive" number of positive charges (Segalla et al., 2002). On the basis of this theory, a tetra-cationic zinc phthalocyanine bearing four aminoalkylated peripheral substitutes has been developed and shown to inactivate multi-drug-resistant strains of *C. albicans*. Phthalocyanine-based photodynamic killing of yeasts does not select resistant strains and the treatment is much more toxic to yeast cells than keratinocytes. Mutagenic effects have not been demonstrated with *C. albicans* or *K. marxianus*.

In the only study published to date on the ability of phthalocyanines to kill dermatomycetes by photodynamic action, lipophilic phthalocyanines were found to exhibit only a fungistatic effect against *T. rubrum* following irradiation. This effect lasted only about 1 week (Smijs and Schuitmaker, 2003).

Drug delivery

As with antibacterial PACT, the majority of published antifungal PACT studies have concentrated on *in vitro* investigations aimed at elucidating photosensitiser and light doses effective in the photodynamic killing of yeasts or dermatomycetes. However, in order to move PACT from the laboratory into the clinic, drug delivery systems must be formulated to enable photosensitiser delivery to the site of infection. Investigations must also be done to determine the likely clinical performance of such delivery systems. To date, only a very small number of studies of this type have been published.

Donnelly et al. (2005) studied the *in vitro* penetration of ALA across human nail and into neonate porcine hoof when released from a novel bioadhesive patch containing 50 mg cm^{-2} ALA. The authors proposed that, if sufficient concentrations of ALA could be achieved within the nail matrix and at the nail bed, PACT may prove to be a useful treatment for onychomycosis. Patch application for 24 h allowed an ALA concentration of 2.8 mM to be achieved on the ventral side of excised human nail. Application for 48 h induced a concentration of 6.9 mM. Application time had no significant effect on the ALA concentration at mean depths of 2.375 mm in neonate porcine, with application times of 24, 48 and 72 h all producing concentrations of 0.1 mM. Incubation of *C. albicans* and *T. interdigitale* with ALA concentrations of 10.0 mM for 30 min and 6 h, respectively, caused reductions in viability of 87% and 42%, respectively, following irradiation with red light. Incubation with a lower concentration of 0.1 mM ALA for 30 min and 6 h, respectively, caused reductions in viability of 32% for *C. albicans* and 6% for *T. interdigitale*, following irradiation. These findings led the authors to suggest that ALA penetration across nail may have to be improved using penetration enhancers, or by filing of the relatively impenetrable dorsal surface of the nail. Alternatively, iron chelators could be used to increase PpIX production for a given ALA dose. The authors concluded that, with such suitable modifications, ALA-based PACT may prove to be a viable alternative in the treatment of onychomycosis.

Donnelly et al. (2007) also reported on a mucoadhesive patch containing TBO, as a potential delivery system for use in PACT of oropharyngeal candidiasis. Patches prepared from aqueous blends of poly(methyl vinyl ether/maleic anhydride) and tripropyleneglycol methyl ether possessed suitable properties for use as mucoadhesive drug delivery systems and were capable of resisting dissolution when immersed in artificial saliva. When releasing directly into an aqueous sink, patches containing 50 and 100 mg TBO cm^{-2} both generated receiver compartment concentrations exceeding the concentration (2.0–5.0 mg ml^{-1}) required to produce high levels of kill (>90%) of both planktonic and biofilm-grown *C. albicans* upon illumination (635 nm, 100–200 J cm^{-2}). However, the concentrations of TBO in the receiver compartments separated from patches by membranes intended to mimic biofilm structures were an order of magnitude below those inducing high levels of kill, even after 6 h release. The authors concluded that short application times of TBO-containing mucoadhesive patches would only allow effective treatment of recently acquired oropharyngeal candidiasis, caused solely by planktonic cells. Longer patch application times may, however, be required for persistent disease where biofilms are implicated.

Smijs et al. (2007) used an *ex vivo* human skin model to investigate the ability of porphyrins to kill *T. rubrum*. The photosensitisers, in liquid vehicles, were applied to the skin, which had been previously inoculated with the dermatomycete. It was found that short incubation times (8 h) gave complete kill upon irradiation (108 J cm^{-2}, 580–870 nm), while incubation for longer times (>24 h) prior to irradiation yielded no kill. Water was a more effective delivery vehicle, in terms of kill rate, than the cell culture medium DMEM.

Light delivery

By definition, PACT requires a source of light to supply the requisite energy for singlet oxygen

production *in situ*. The energy required is determined by the molecular structure of the photosensitiser and, thus, a different light excitation range is required for the phenothiaziniums (ca. 600–660 nm) than for the phthalocyanines (ca. 630–690 nm). Ideally light sources should provide a strong output at the requisite wavelength for photoexcitation. Lasers, and the less expensive and easier to use, filtered incoherent lamps are the most commonly employed sources in PACT today. White or fluorescent light sources may be used. However, for *in vivo* use, emission in the ultraviolet range should be minimised, due to the risk of mutagenesis. Similarly, emission in the infrared range is also undesirable, so as to avoid heating of tissue. Typical power outputs for light sources used in antifungal PACT are in the range 10–100 mW cm^{-2}, with typical total light doses being between 10 and 200 J cm^{-2}. In some cases, these may need to be higher than those used in antibacterial PACT in order to yield comparable rates of kill (Donnelly et al., 2007).

Light fluence through tissue decreases exponentially with thickness. This decrease is determined by absorption, particularly by haemoglobin, and scattering, parameters that vary between tissue types (Brancaleon and Moseley, 2002). Due to the inability of light to penetrate deeply into tissue, clinical PACT is necessarily limited to areas of the body that can be irradiated from the surface. Thus, antifungal treatment would be restricted to infections of the skin, nails, hair, oral cavity, oesophagus and lower female reproductive tract. In treating such infections, however, some degree of tissue penetration is required to, for example, kill fungi residing below the surface of the skin or in the matrix of the nail. Light in the red region of the spectrum penetrates tissue down to around 3.0 mm, while light in the blue region penetrates down to only around 1.5 mm. Thus, the porphyrins are typically excited by light in the red region of the spectrum, rather than blue light, which they absorb more efficiently (Gannon and Brown, 1999). Consequently, much work has been devoted to the phthalocyanines, which absorb more effectively at longer wavelengths.

Endogenous light absorption is important in clinical applications of antifungal PACT. It is essential that photosensitisers used to kill fungi can be photoexcited and this will not occur if the incident light is absorbed by fungal pigment. Thus, photosensitisers absorbing beyond the range of the pigment are required, with appropriate light sources. As with all proposed protocols, a thorough knowledge of the photoproperties of both target and agent will be essential.

In vivo studies

The interest raised by the promising *in vitro* findings reported to date has led to a small number of *in vivo* studies. 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 (Teichert et al., 2002). Effects were dependent on light and drug doses. The authors concluded that MB-PDT might represent an effective, non-toxic, simple, inexpensive and repeatable therapy of oral Candidiasis in immunodeficient HIV/AIDS patients which, as is well known, often select resistant fungal strains. However, the therapeutical technique used has the notable limitation of not covering the oesophageal infection, which very often occurs concomitantly in such patients. In addition, the use of an aqueous solution is not practical and would lead to significant and undesirable blue staining of teeth and mucous membranes. A further drawback of this study is that results of follow-up examinations were not reported, meaning no evidence of long-term cure was provided. Therefore, the patch-based system described by Donnelly et al. (2007) may be more suitable for this application.

The clinical efficacy of ALA-PACT in the treatment of fungal infections of human skin has been investigated with an open pilot study enrolling nine patients with interdigital mycosis of the feet (Calzavara-Pinton et al., 2004). 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 an aqueous solution containing 20% w/w ALA and irradiation with UV light, indicating PpIX production. The treatment protocol used consisted of the application of a 20% w/w ALA cream under an occlusive dressing, followed, 4 h later, by the irradiation of 75 J cm^{-2} of broadband red light. This protocol was similar to that employed in photodynamic treatment of neoplastic lesions of the skin. 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. A low level of effectiveness was observed overall and a high percentage of patients exhibited localised

erythema, oedema and desquamation 3–5 days following PACT. This effect of PACT in local fungal infection may have resulted from destruction to the whole epidermis infected with mycosis causative organisms.

The less than satisfactory results obtained could possibly be explained by insufficient ALA sensitization of fungi *in vivo* because of different temperature, humidity and pH conditions from laboratory research. However, *in vivo* applications of PACT should always take into consideration the phototoxic effect on the surrounding environment, such as on keratinocytes and cells of the immune system. Improper selection of a photosensitizer can lead to high phototoxic activity against the causative agent *in vitro*. However, the simultaneous killing of skin cells can also occur. Therefore, instead of eradicating the infecting fungi, there will be damage to the protective *stratum corneum* barrier and significant formation of cellular debris as a result of cell death in the surrounding tissue. Obviously, this will then provide a favourable background for further microbial infection.

The problems associated with this study are largely due to the accumulation of the photosensitiser PpIX in both fungal and human cells following topical ALA application. As described previously, saturation of the normal haem biosynthetic pathway will lead to PpIX accumulation in human cells with subsequent damage upon irradiation. The use of a preformed photosensitiser that does not accumulate to a great extent in human cells may have produced a better result. However, even though such agents could be used in a clinical trial on a named-patient basis, they would have to be prepared to good manufacturing practice (GMP) standards. As such, any clinical investigations of PACT in humans will be limited to those employing commercially available photosensitisers. Most of these drugs have been designed specifically for use in the related anticancer PDT discipline. As such, these agents do not necessarily possess the ideal physicochemical characteristics for use in PACT. Therefore, until a wider range of GMP-standard cationic porphyrins and phthalocyanines become commercially available, human clinical trials of antifungal PACT are likely to be limited to those based on phenothiaziniums, such as MB and TBO.

Conclusion

Results of experimental investigations have demonstrated conclusively that both dermatomycetes and yeasts can be effectively killed by photodynamic action employing phenothiazinium, porphyrin and phthalocyanine photosensitisers. Importantly, considerable selectivity for fungi over human cells has been demonstrated, no reports of fungal resistance exist and the treatment is not associated with genotoxic or mutagenic effects to fungi or human cells. In spite of the success of cell culture investigations, only a very small number of *in vivo* animal and human trials have been published. Photodynamic therapy has the potential to evolve into a useful treatment for difficult to eradicate fungal infections of accessible regions of the body. For example, the prospect of eradicating oral thrush in an AIDS patient, or denture stomatitis in an elderly nursing home resident in a single session, or a once-off curative treatment for onychomycosis, is a scenario that would be attractive to both patients and health service providers. However, this will not become a clinical reality until pharmaceutical companies and grant-awarding bodies devote considerable resources to the development of both photosensitisers specifically designed for antifungal treatment and drug delivery systems that allow such agents to be efficiently delivered to their sites of action.

References

Berg K, Bommer JC, Moan J. Evaluation of sulfonated aluminium phthalocyanines for use in photochemotherapy. A study on the relative efficiencies of photoinactivation. Photochem Photobiol 1989;49: 587–94.

Bertoloni G, Rossi G, Valduga G, Jori G, Ali H, Van Lier JE. Photosensitisng activity of water- and lipid-soluble phthalocyanines on prokaryotic and eukaryotic microbial cells. Microbios 1992;71:33–46.

Bertoloni G, Sacchetto R, Jori G, Vernon DJ, Brown SB. Protoporphyrin photosensitisation of *Enterococcus hirae* and *Candida albicans* cells. Las Life Sci 1993:267–75.

Bertoloni G, Zambotto F, Conventi L, et al. Role of specific cellular targets in the hematoporphyrin-sensitized photoinactivation of microbial-cells. Photochem Photobiol 1987;46(5):695–8.

Blignaut E, Molepo J, Pujol C, Soll DR, Pfaller MA. Clade-related amphotericin B resistance among South African *Candida albicans* isolates. Diagn Microbiol Infect Dis 2005;53:29–31.

Bliss JM, Bigelow CE, Foster TH, Haidaris CG. Susceptibility of *Candida* species to photodynamic effects of Photofrin®. Antimicrob Agent Chemother 2004;48: 2000–6.

Bonnet R. Photosensitisers of the porphyrin and phthalocyanine series for photodynamic therapy. Chem Soc Rev 1995;24:19–33.

Bonnett R, Djelal BD, Nguyen A. Physical and chemical studies related to the development of m-THPC

(Foscan®) for the photodynamic therapy (PDT) of tumours. J Porph Phthalocyan 2001;5:652–61.
Brancaleon L, Moseley H. Laser and non-laser light sources for photodynamic therapy. Laser Med Sci 2002;17:173–86.
Brouillet N, Arselin-De Chateaubodeau G, Volland C. Stiudies on protoporrphyrin biosynthetic pathway in *Saccaromyces cerevisiae*: Characteristization of the tetrapyrrole intermediates. Biochimie 1975;57: 647–55.
Calzavara-Pinton PG, Venturini M, Capezzera R, Sala R, Zane C. Photodynamic therapy of interdigital mycoses of the feet with topical application of 5-aminolevulinic acid. Photodermatol Photoimmunol Photomed 2004;20:144–7.
Carré V, Gaud O, Sylvain I, Bourdon O, Spiro M, Blais J, et al. Fungicidal properties of meso-arylglycosylporphyrins: Influence of sugar substituents on photoinduced damage in the yeast *Saccharomyces cerevisiae*. J Photochem Photobiol B: Biol 1999;48:57–62.
Chabrier-Roséllo Y, Foster TH, Pérez-Nazario N, Mitra S, Haidaris CG. Sensitivity of *Candida albicans* germ tubes and biofilms to Photofrin®-mediated phototoxicity. Antimicrob Agent Chemother 2005;49:4288–95.
Codling CE, Maillard JY, Russell AD. Aspects of the antimicrobial mechanisms of action of a polyquaternium and an amidoamine. J Antimicrob Chemother 2003;51:1153–8.
Cohen PR, Scher RK. Topical and surgical treatment of onychomycosis. J Am Acad Dermatol 1994;31:S74–7.
De Rosa FS, Bentley MVLB. Photodynamic therapy of skin cancers: sensitizers, clinical studies and future directives. Pharm Res 2000;17:1447–55.
De Souza SC, Junqueira JC, Balducci I, Koga-Ito CY. Photosensitisation of different Candida species by low power laser light. J Photochem Photobiol B: Biol 2006;83:34–8.
Demidova TN, Hamblin MR. Effect of cell-photosensitizer binding and cell density on microbial photoinactivation. Antimicrob Agents Chemother 2005;49:2329–35.
Denning DW. Can we prevent azole resistance in fungi? Lancet 1995;346:454–5.
Dodgson AR, Dodgson KJ, Pujol C, Pfaller MA, Soll DR. Clade-specific flucytosine resistance is due to a single nucleotide change in the FUR1 gene of *Candida albicans*. Antimicrob Agents Chemother 2004;48:2223–7.
Donnelly RF, McCarron PA, Lightowler JM, Woolfson AD. Bioadhesive patch-based delivery of 5-aminolevulinic acid to the nail for photodynamic therapy of onychomycosis. J Control Rel 2005;103:381–92.
Donnelly RF, McCarron PA, Tunney MM, Woolfson AD. Potential of photodynamic therapy in treatment of fungal infections of the mouth. Design and characterisation of a mucoadhesive patch containing toluidine blue O. J Photochem Photobiol B: Biol 2007;86:59–69.
Dougherty TJ, Gomer CJ, Henderson BW, Jori G, Kessel D, Korbelik M, et al. Photodynamic therapy. J Natl Cancer Inst 1998;90:889–905.
Eggimann P, Garbino J, Pittet D. Epidemiology of *Candida* species infections in critically ill non-immunocompromised patients. Lancet Infect Dis 2003;3: 685–702.
Fraikin GY, Strakhovskaya MG, Rubin AB. The role of membrane-bound porphyrin-type compound as endogenous sensitiser in photodynamic damage to yeast plasma membranes. J Photochem Photobiol B: Biol 1996;34:129–35.
Gannon MJ, Brown SB. Photodynamic therapy and its applications in gynaecology. Br J Obstet Gynaecol 1999;106:1246–54.
Hay RJ. New developments in antifungals. Int J Dermatol S 1999;2:65–9.
Isaacs NS. Physical organic chemistry. Essex, UK: Longman Scientific and Technical; 1992.
Ito T. Photodynamic action of haematoporphyrin on yeast cell: a kinetic approach. Photochem Photobiol 1981;34:521–4.
Johnson EM, Warnock DW, Luker J, Porter SR, Scully C. Emergence of azole drug-resistance in Candida species from HIV-infected pateients receiving prolonged fluconazole therapy for oral candidiasis. J Antimicrob Chemother 1995;35:103–14.
Jori G, Brown SB. Photosensitized inactivation of microorganisms. Photochem Photobiol Sci 2004;3:403.
Kalka K, Merk H, Mukhtar H. Photodynamic therapy in dermatology. J Am Acad Dermatol 2000;42:389–413.
Kalyanasundaram K. Photochemistry of polypyridine and porphyrin complexes. London, UK: Academic Press; 1992.
Kamp H, Tietz HJ, Lutz M, Piazana H, Sowyrda P, Lademann J, et al. Antifungal effect of 5-aminolevulinic acid PDT in *Trichophyton rubrum*. Mycoses 2005;48:101–7.
Katz HI. Possible drug interactions in oral treatment of onychomycosis. J Am Acad Dermatol 1997;87:571–3.
Kibbler CC, Seaton S, Barnes RA, Gransden WR, Hollimann RE, Johnson EM, et al. Management and outcome of bloodstream infections due to *Candida* species in England and Wales. J Hosp Infect 2003;54:18–24.
Konan YN, Gurny R, Allemann E. State of the art in the delivery of photosensitizers for photodynamic therapy. J Photochem Photobiol B: Biol 2002;66:89–106.
Lambrechts SAG, Aalders MCG, Verbraak FD, Lagerberg JWM, Dankert JB, Schuitmaker JJ. Effect of albumin on the photodynamic inactivation of microorganisms by a cationic porphyrin. J Photochem Photobiol B: Biol 2005a;79:51–7.
Lambrechts SAG, Schwartz KR, Aalders MCG, Dankert JB. Photodynamic inactivation of fibroblasts by a cationic porphyrin. Lasers Med Sci 2005b;20:62–7.
Malik Z, Ladan H, Nitzan Y. Photodynamic inactivation of Gram-negative bacteria: problems and possible solutions. J Photochem Photobiol B: Biol 1992;14:261–6.
Moan J. On the diffusion length of singlet oxygen in cells and tissues. J Photochem Photobiol B: Biol 1990; 6:343–7.
Moan J, Peng Q. An outline of the hundred-year history of PDT. Anticancer Res 2003;23:3591–600.
Moan J, Streckyte G, Bagdonas S, Bech O, Berg K. Photobleaching of protoporphyrin IX in cells

incubated with 5-aminolevulinic acid. Int J Cancer 1997;70:90–7.

Monfrecola G, Procacci EM, Bevilacqua M, Manco A, Calabro G, Santoianni P. *In vitro* effects of 5-aminolaevulinic acid plus visible light on *Candida albicans*. Photochem Photobiol Sci 2004;3:419–22.

Moretti B, Correa S, Battle A. Porphyrin biosynthesis intermediates are not regulating delta-aminolevulinic acid transport in *Saccaromyces cerevisiae*. Biochem Biophys Res Commun 2000;272:946–50.

Nikaido H. Permability of the lipid domains of bacterial membranes. In: Aloia RC, Curatin CVC, Gordon LM, editors. Membrane transport and information storage. New York, USA: Alan R. Liss; 1990. p. 165–90.

Oschner M. Photophysical and photobiological processes in the photodynamic therapy of tumours. J Photochem Photobiol B: Biol 1997;39:1–18.

Paardekopper M, van de Broek PJ, de Bruijne AW, Elferink JG, Dubbelman TM, van Steveninck J. Photodynamic treatment of yeast cells with the dye toluidine blue: all-or-none loss of plasma membrane barrier properties. Biochim Biophys Acta 1992;1108:86–90.

Paardekopper M, Van Gompel AE, De Boont HJGM, Nagelkerke JF, Van Stevenick J, Van der Broek PJA. Photodynamic treatment of yeast with chloroaluminium-phthalocyanine: role of the monomeric form of the dye. Photochem Photobiol 1994;59:161–6.

Paardekopper M, van Gompel AE, van Steveninck J, van de Broek J. The effect of photodynamic treatment of yeast with the sensitiser chloroaluminium phthalocyanine on various cellular parameters. J Photochem Photobiol B: Biol 1995;62:561–7.

Pankhurst C. Oropharyngeal candidiasis. Clin Evid 2001;4:761–72.

Peng Q, Berg K, Moan J, Kongshaug M, Nesland JM. 5-Aminolevulinic acid-based photodynamic therapy: principles and experimental research. Photochem Photobiol 1997;65:235–51.

Pujol C, Pfaller MA, Soll DR. Flucytosine resistance is restricted to a single genetic clade of *Candida albicans*. Antimicrob Agents Chemother 2004;48:262–6.

Romagnoli C, Mares D, Sacchetti G, Bruni A. The photodynamic effect of 5-(4-hydroxy-1-butinyl)-2,2-biethienyl on dermatomycetes. Mycol Res 1998;102:1519–24.

Segalla A, Borsarelli CD, Braslavsky SE, Spikes JD, Ronucci G, Dei D, et al. Photophysical, photochemical and antibacterial photosensitising properties of a novel octacationic Zn(II)-phthalocyanine. Photochem Photobiol Sci 2002;1:641–8.

Smijs TGM, Schuitmaker HJ. Photodynamic Inactivation of the dermatomycete *Trichophyton rubrum*. Photochem Photobiol 2003;77:556–60.

Smijs TGM, van der Haas RNS, Lugtenburg J, Liu Y, de Jong RLP, Schuitmaker HJ. Photodynamic treatement of the dermatomycete *Trichophyton rubrum* and its microconidia with porphyrin photosensitisers. Photochem Photobiol 2004;80:197–202.

Smijs TGM, Bouwstra JA, Schuitmaker HJ, Talebi M, Pavel S. A novel ex vivo skin model to study the susceptibility of the dermatomycete *Trichophyton rubrum* to photodynamic treatment in different growth phases. J Antimicrob Chemother 2007;59:433–40.

Strakhovskaya MG, Shumarina AO, Fraikin GY, Rubin AB. Synthesis of protoporphyin IX induced by 5-aminolevulinic acid in yeast cells in the presence of 2,2-dipyridyl. Biochem Moscow 1998;63:859–63.

Teichert MC, Jones JW, Usacheva MN, Biel MA. Treatment of oral candidiasis with methylene blue-mediated photodynamic therapy in an immunodeficient murine model. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2002;93:155–60.

Wainwright M. Photodynamic antimicrobial chemotherapy. J Antimicrob Chemother 1998;42:13.

Wainwright M, Crossley B. Photosensitising agents – circumventing resistance and breaking down biofilms: a review. Int Biodeter Biodegrad 2004;53:119.

Walsh TJ, Viviani MA, Arathoon E, Chiou C, Ghannoum M, Groll AH, et al. New targets and delivery systems for antifungal therapy. Med Mycol 2000;38(Suppl. 1): 335–47.

Wilson M, Mia N. Sensitisation of *Candida albicans* to killing by low-power laser light. J Oral Pathol Med 1993;22:354–7.

Zeina B, Greenman J, Purcell WM, Das B. Killing of cutaneous microbial species by photodynamic therapy. Br J Dermatol 2001;144:274–8.

Zeina B, Greenman J, Corry D, Purcell WM. Cytotoxic effects of antimicrobial photodynamic therapy on keratinocytes *in vitro*. Br J Dermatol 2002;146: 568–73.

Zeina B, Greenman J, Corry D, Purcell WM. Antimicrobial photodynamic therapy: assessment of genotoxic effects on keratinocytes *in vitro*. Br J Dermatol 2003;148: 229–32.

Zoladek T, Nguyen BN, Jagiello I, Graczyk A, Rytka J. Diamino acid derivatives of porphyrins penetrate into yeast cells, induce photodamage, but have no mutagenic effects. Photochem Photobiol 1997;66: 253–9.