RESEARCH ARTICLE

Susceptibility of *Candida albicans* to photodynamic action of 5,10,15,20-tetra(4-*N*-methylpyridyl)porphyrin in different media

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Abstract

The photodynamic activity of 5,10,15,20-tetra(4-*N*-methylpyridyl)porphyrin (TMPyP) was evaluated *in vitro* on *Candida albicans* cells under different experimental conditions. This tetracationic porphyrin binds rapidly to *C. albicans* cells, reaching a value of $\sim$1.7 nmol 10^{-6} cells when the cellular suspensions (10^6 CFU mL^{-1}) were incubated with 5 μM sensitizer. The amount of cell-bound sensitizer is not appreciably changed when cultures are incubating for longer times ($>$ 15 min) but it diminishes with the number of washing steps. Photosensitized inactivation of *C. albicans* cellular suspensions increases with both sensitizer concentration and irradiation time, causing a $\sim$5 log decrease of cell survival when the cultures are treated with 5 μM TMPyP and irradiated for 30 min. However, the photocytotoxicity decreases after one washing step, with the decrease in cell-bound sensitizer. The growth of *C. albicans* cells was arrested when the cultures were exposed to 5 μM TMPyP and visible light. On agar surfaces, the phototoxic effect of this sensitizer, which caused an inactivation of *C. albicans* cells, remained high. No growth was observed in areas containing TMPyP and irradiated. Moreover, in small *C. albicans* colonies, *C. albicans* cells were completely inactivated. These studies indicate that TMPyP is an effective sensitizer for photodynamic inactivation of yeasts in both liquid suspensions or localized on a surface.

Introduction

The increasing diffusion of infectious diseases represents a major challenge for human health worldwide, especially as a consequence of the continuous emergence of antifungal-resistant yeasts. The ubiquitous commensal member of the human microbial communities, *Candida albicans*, an opportunistic pathogen, is the most important human fungal pathogen, causing various diseases, from superficial mucosal infections to life-threatening systemic disorders (Bliss *et al.*, 2004; Fidel, 2006; Munin *et al.*, 2007). Conventional treatments for oral candidiasis have been shown to have a fungistatic rather than a fungicidal effect, resulting in inadequate treatment of patients. In addition, the increasing resistance of *C. albicans* to both systemic and topical antifungal agents has made effective treatment more difficult (Goldman *et al.*, 2004; Brion *et al.*, 2007; Ribeiro & Rodrigues, 2007). Therefore, therapeutic alternatives based on different strategies are required. Photodynamic therapy, initially proposed for the treatment of tumors, has shown numerous nononcologic applications. At present, this procedure has several useful biological effects, with use in photodynamic insecticides (Ben Amor & Jori, 2000), photodynamic antibacterial, antifungal and antiviral agents (Wainwright, 2004) and photosterilization of water, biological fluids and blood contaminated with pathogenic microorganisms (Jori & Brown, 2004). In this sense, photodynamic inactivation (PDI) with positively charged photosensitizing agents appears to be a promising approach, especially for the treatment of fungal infections (Calzavara-Pinton *et al.*, 2005). This methodology was also proposed as a new antifungal therapy for the treatment of onychomycosis (Kumar & Kimball, 2009). PDI utilizes visible light to activate a photosensitizer, which can react with molecules in its direct environment by electron or hydrogen transfer, leading to the production of radicals (type I reaction). It can also transfer its energy to oxygen, generating the highly reactive singlet molecular oxygen, $O_2(^1\Delta_g)$ (type II reaction). Both pathways can cause cell damage that inactivates the microorganisms (Hamblin & Hasan, 2004; Jori &

Brown, 2004; Durantini, 2006). PDI has several advantages for the treatment of infections caused by microbial pathogens, including a broad spectrum of action, efficient inactivation of antibiotic-resistant strains, low mutagenic potential and the lack of selection of photoresistant microbial cells.

The fungal cell wall gives structure to the cell and protects it from the environment (Osumi, 1998). Therefore, the efficiency of the photosensitized process is markedly more pronounced when used in agents to enhance their penetration into the inner cell area. Recently, cationic porphyrin derivatives have been investigated for PDI applications in the treatment and control of yeasts (Lambrechts *et al.*, 2005a, b; Cormick *et al.*, 2009). The effects of different medium conditions during PDI were studied using methylene blue and toluidine blue on *C. albicans* (Carvalho *et al.*, 2009). The effects of natural product extracts from *Alternanthera maritima* on the viability of *Candida dubliniensis* (Gasparetto *et al.*, 2010) were also studied. In general, far more systematic studies have been carried out on effective photosensitizers to eradicate bacteria than to eradicate various species of yeast and fungi. One of the most active sensitizers established to inactivate bacteria is 5,10,15,20-tetra(4-*N*-methylpyridyl)porphyrin (TMPyP) (Merchat *et al.*, 1996a, b; Valduga *et al.*, 1999; Salmon-Divon *et al.*, 2004). However, there are very few organized investigations of PDI using this photosensitizer.

In this work, the photodynamic action of TMPyP to eliminate *C. albicans* was evaluated under different conditions. The results for TMPyP were compared with those previously obtained for 5,10,15,20-tetra(4-*N*,*N*,*N*-trimethylammoniumphenyl)porphyrin (TMAP), which is a standard active sensitizer that has been established to eradicate *C. albicans* (Cormick *et al.*, 2009). These studies determined the conditions for eradication of yeast cells by TMPyP in cell suspensions, in cell localized on agar and in colonies immobilized on surfaces.

Materials and methods

General

Absorption and fluorescence spectra were recorded on a Shimadzu UV-2401PC spectrometer and a Spex FluoroMax fluorometer, respectively. Spectra were recorded using 1-cm path length quartz cuvettes at 25.0 ± 0.5 °C. Culture absorption was determined at 660 nm in a Barnstead Turner SP-830 (Dubuque, IA) spectrophotometer. The light source used was a Novamat 130 AF (Braun Photo Technik, Nürnberg, Germany) slide projector equipped with a 150 W lamp. The light was filtered through a 2.5-cm glass cuvette filled with water to absorb heat. A wavelength between 350 and 800 nm was selected by optical filters. The fluence rate at the treatment site was 90 mW cm^{-2} (Radiometer Laser Mate-Q, Coherent, Santa Clara, CA).

All the chemicals were from Aldrich (Milwaukee, WI) and were used without further purification. Solvents (GR grade) from Merck (Darmstadt, Germany) were distilled. Ultrapure water was obtained from Labconco (Kansas, MO) equipment model 90901-01.

Sensitizer

5,10,15,20-Tetra(4-*N*-methylpyridyl)porphyrin *p*-tosylate (TMPyP) was purchased from Aldrich. A porphyrin stock solution (~0.5 mM) was prepared by dissolution in 1 mL of water. The concentration was checked by spectroscopy, taking into account the value of the molar coefficient (Merchat *et al.*, 1996b).

Microorganism and growth conditions

The strain of *C. albicans* PC31, recovered from human skin lesion, was previously characterized and identified (Cormick *et al.*, 2009). Yeast was grown aerobically overnight in Sabouraud broth (Britania, Buenos Aires, Argentina) (3 mL) at 37 °C to stationary phase. An aliquot of this culture (1 mL) was dissolved in 3 mL Sabouraud broth. Cells were then harvested by centrifugation of broth cultures (1200 ***g*** for 15 min) and resuspended in 4 mL of 10 mM phosphate-buffered saline (PBS, pH = 7.0), corresponding to ~10^7 CFU mL^{-1}. The cells were appropriately diluted to obtain ~10^6 CFU mL^{-1} in PBS. In all the experiments, 2 mL of the cell suspensions in Pyrex® brand culture tubes (13 × 100 mm) was used and the sensitizer was added from a stock solution of ~0.5 mM in water. Viable *C. albicans* cells were monitored and the number of CFU was determined on Sabouraud agar plates after ~48-h incubation at 37 °C. Fungal cultures grown under the same conditions with or without photosensitizers kept in the dark and illuminated cultures without sensitizer served as controls.

Photosensitizer binding to yeast cells

Suspensions of *C. albicans* (2 mL, ~10^6 CFU mL^{-1}) in PBS were incubated in the dark at 37 °C with a set concentration (1, 5 and 10 μM) of TMPyP for different times. The sensitizer was added from a 0.5 mM stock solution in water. The cultures were centrifuged (1200 ***g*** for 15 min) and the cell pellets resuspended in 2% aqueous sodium dodecyl sulfate (SDS) 2 mL, incubated overnight at 4 °C and sonicated for 30 min. The concentration of sensitizer in the supernatant was measured by spectrofluorimetry ($\lambda_{exc} = 425$ nm, $\lambda_{em} = 656$ nm) in a solution of 2% SDS in PBS. The fluorescence values obtained from each sample were in reference to the total number of cells contained in the suspension. The concentration of the porphyrin in this sample was estimated by comparison with a calibration

curve obtained with standard solutions of the TMPyP in 2% SDS (sensitizer ~0.005–0.1 μM).

Photosensitized inactivation of *C. albicans* cells in PBS

Cellular suspensions of *C. albicans* (2 mL, $\sim 10^6$ CFU mL^{-1}) in PBS were incubated with an appropriate concentration (1, 5 and 10 μM) of porphyrin for 30 min in the dark at 37 °C. TMPyP was added from a 0.5 mM stock solution in water. After that, the cultures were exposed to visible light for different time intervals. Control experiments were carried out without illumination in the absence and in the presence of sensitizer. Control and irradiated cell suspensions were serially diluted with PBS, each solution was plated in triplicate on Sabouraud agar and the number of colonies formed after ~48-h incubation at 37 °C was counted.

Growth delay of *C. albicans*

Cultures of *C. albicans* cells were grown overnight as described above. A portion (1 mL) of this culture was transferred to 20 mL of fresh Sabouraud broth medium. The suspension was homogenized and aliquots of 2 mL were incubated with 5 μM of sensitizer at 37 °C. The culture grown was measured by turbidity at 660 nm using a Tuner SP-830 spectrophotometer. The flasks were then irradiated with visible light at 37 °C, as described above. In all cases, control experiments were carried out without illumination in the absence and in the presence of sensitizer.

TMPyP uptake in solution and photosensitized inactivation of *C. albicans* cells on agar

Suspensions of *C. albicans* (2 mL, $\sim 10^6$ CFU mL^{-1}) in PBS were incubated 30 min in the dark at 37 °C with different concentrations of sensitizer (1, 2.5 and 5 μM). The cells were diluted 1/1000 in PBS and 100 μL of the cultures were spread on 5-cm-diameter Sabouraud agar plates. The cultures were incubated for 30 min and the plates then irradiated for 30 min with visible light as described above. The number of colonies formed after 48-h incubation at 37 °C was counted. *Candida albicans* cellular viability in this medium was determined for cultures treated with 5 μM TMPyP at different irradiation times. Control experiments with and without photosensitizers kept in the dark as well as illuminated controls without porphyrin were carried out.

TMPyP uptake and photosensitized inactivation of *C. albicans* cells on agar

Sabouraud agar plates (5 cm diameter) were spread on an area of ~0.6 cm^2 with different amounts of TMPyP (2.2, 4.5, 6.7 and 9.0 nmol) from a 0.5 mM solution in water. Plates were spread with the suspension of *C. albicans* ($\sim 10^6$ CFU mL^{-1}) in PBS. The plates were then incubated at 37 °C for 30 min in the dark. After that, the plates were irradiated as described above for 30 min and incubated for 48 h at 37 °C in the dark. Plates with or without photosensitizers kept in the dark and irradiated plates without porphyrin were used as controls.

Photosensitization of *C. albicans* growing in colonies on agar

Suspensions of *C. albicans* ($\sim 10^2$ CFU mL^{-1}) in PBS were spread on 10-cm-diameter plates containing Sabouraud agar and grown at 37 °C for 24 h. About 15 small colonies per plate were obtaining using this procedure. The colonies were spread with 9.0 nmol TMPyP from a 0.5 mM stock solution in water. The cultures were kept in the dark for 30 min at 37 °C to allow the binding of sensitizer to *C. albicans* cells and irradiated with visible light from a slide projector as described above or from direct natural sunlight at midday (~80 mW cm^{-2}). The plates were then incubated in the dark at 37 °C and the sizes of the *C. albicans* colonies were determined at different times. The viability of cells after PDI treatment was evaluated by transferring samples of these colonies to fresh Sabouraud broth and plating on a new Sabouraud agar plate. Control experiments with or without photosensitizers kept in the dark as well as illuminated controls without porphyrin were carried out.

Statistical analysis

All data are presented as the mean ± SD of each group. Variation between groups was evaluated using Student's *t*-test; a confidence level of 95% ($P < 0.05$) was considered statistically significant. Each experiment was repeated separately three times.

Results

Binding of TMPyP to *C. albicans* cells

The capacity of TMPyP to bind to *C. albicans* cells was determined in cell suspensions of $\sim 10^6$ cells mL^{-1} in PBS. Thus, the *C. albicans* cultures were incubated with 5 μM TMPyP for different periods of time at 37 °C in the dark. The amount of porphyrin recovered after different incubation periods is shown Fig. 1. TMPyP reached the highest value of cell-bound sensitizer after a short time (< 15 min). An increase in the amount of photosensitizer bound to *C. albicans* cells was not observed when the incubation time was extended to 30 min. The results shown in Fig. 1 indicate that the TMPyP binding led to a saturation value of 1.70 nmol 10^{-6} cells.

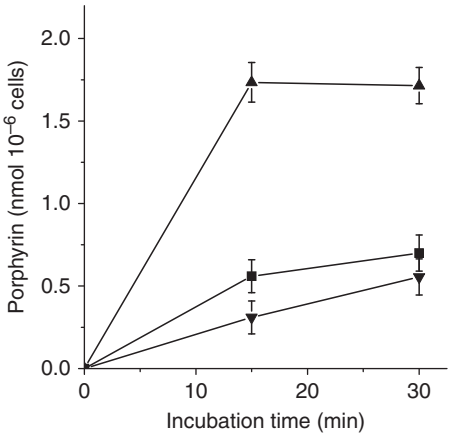

Fig. 1. Amount of TMPyP recovered from *Candida albicans* cells ($\sim 10^6$ CFU mL^{-1}) treated with 5 μM of sensitizer at 37 °C in the dark for different incubation times and after different washing steps; 0 (▲), 1 (■) and 2 (▼). Values represent mean ± SD of three separate experiments.

The recovery of TMPyP from *C. albicans* cells was determined after different washing steps, varying the incubation times (Fig. 1). The amount of cell-bound TMPyP decreased when the cells were washed with PBS, although the decrease was less after the first washing step. There was also a trend for the amount of porphyrin remaining to increase with incubation time. After 30 min, values of 0.70 and 0.50 nmol 10^{-6} cells were found for cells washed once and twice, respectively, with PBS. The amount of cell-bound porphyrin was evaluated after three and four washed steps, respectively giving 0.38 and 0.33 nmol 10^{-6} cells.

Photosensitized inactivation of *C. albicans* cell suspensions

First, the cell toxicity induced by TMPyP was analyzed in the absence of light at different concentrations of photosensitizer (1, 5 and 10 μM). The results are shown in Fig. 2a. No toxicity was detected for the cells ($\sim 10^6$ cells mL^{-1}) in PBS treated with 1 and 5 μM of sensitizer for 30 min in the dark. However, 10 μM TMPyP was toxic to *C. albicans*, producing a 1.5 log decrease of cell survival. Therefore, 5 μM TMPyP was chosen for experiments in cell suspensions. Control experiments showed that the viability of *C. albicans* was unaffected by illumination alone, indicating that the cell mortality obtained after irradiation of the cultures treated with TMPyP is due to the photosensitization effect of the agent.

PDI of *C. albicans* was evaluated varying TMPyP concentrations (1–5 μM) in PBS cell suspensions after 30-min incubation at 37 °C in the dark (Fig. 2a). The viability of *C. albicans* cells after 30-min irradiation was dependent upon the concentration of porphyrin used in the treatment (Fig. 2a). A 1–5 μM increase in the concentration of sensitizer was

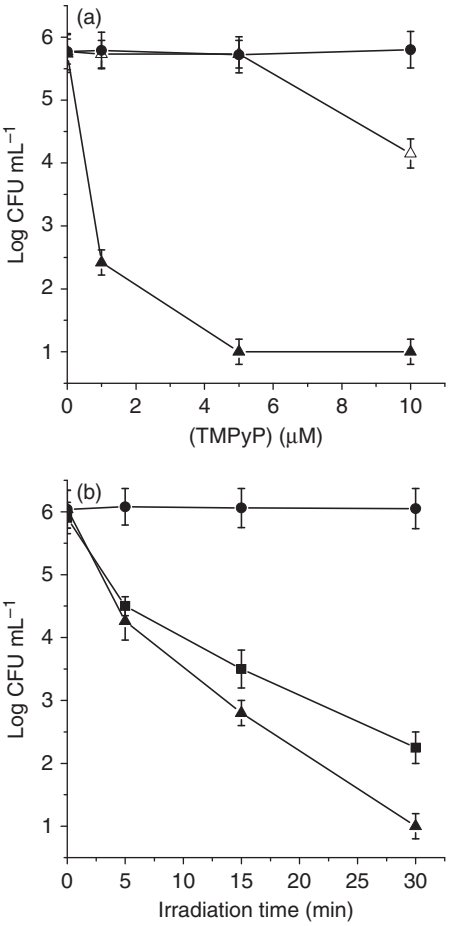

Fig. 2. (a) Survival curves of *Candida albicans* ($\sim 10^6$ CFU mL^{-1}) incubated with different TMPyP concentrations (1, 5 and 10 μM) for 30 min at 37 °C in the dark and exposed to visible light for 30 min (▲) or kept in the dark for 30 min (△). (b) Photoinactivation of *C. albicans* incubated with 5 μM TMPyP for 30 min at 37 °C in the dark and exposed to visible light for different irradiation times without (▲) and with one washing step. Control culture untreated with TMPyP and irradiated (●). Values represent mean ± SD of three separate experiments.

accompanied by an enhancement in PDI efficiency, causing 3.5 log and 5 log decreases, respectively. However, in this range the decrease in cell survival was not linear with concentration. A plateau in the survival plot appears to occur at higher concentrations than 5 μM, when the residual cell survival is extremely low.

The PDI of *C. albicans* treated with 5 μM TMPyP was investigated using different irradiation times (Fig. 2b). The *C. albicans* cells were rapidly photoinactivated when the cultures treated with 5 μM TMPyP were exposed to visible light. Under these conditions, cell inactivation was proportional to the time of irradiation. On the other hand, a decrease in the TMPyP photosensitizing efficiency of ∼1 log after 30-min irradiation was found when cells washed once were irradiated (Fig. 2b).

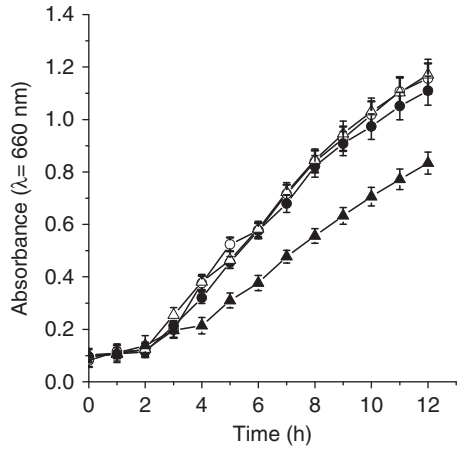

Fig. 3. Growth curves of *Candida albicans* cells incubated with 5 μM TMPyP (▲) and exposed to different irradiation times with visible light in Sabouraud broth at 37 °C. Control cultures: cells treated with 5 μM of TMPyP (△) in the dark, untreated cells irradiated (●) and in the dark (○). Values represent mean ± SD of three separate experiments.

A photocytotoxic action on the growth of *C. albicans* cultures sensitized by TMPyP was seen in Sabouraud medium. TMPyP (5 μM) was added to fresh cultures of *C. albicans* to log phase and the flasks were irradiated with visible light at 37 °C. The effect induced by TMPyP on cell growth is shown in Fig. 3. Cells of *C. albicans* treated with TMPyP in the dark, or not treated with sensitizer and illuminated, showed no growth delay compared with control. Conversely, growth was delayed when *C. albicans* cultures were treated with TMPyP and illuminated.

Photoinactivation of *C. albicans* cells on surfaces

The photodynamic activity of TMPyP was evaluated in *C. albicans* cells immobilized on Sabouraud agar. Two approaches were used to incorporate the sensitizer to the cells. First, the cell suspensions of *C. albicans* in PBS were incubated with different TMPyP concentrations (1, 2.5 and 5 μM) for 30 min at 37 °C in the dark. Then, the cells were plated and the plates incubated for 30 min at 37 °C in the dark. In this experiment, the sensitizer was incorporated in solution and after that the cells were placed on the agar surface. The plates were illuminated for 30 min with visible light. The cell survival after 48 h of incubation in dark is shown in Fig. 4a. As can be observed, no colony formation was observed for cells treated with 5 μM TMPyP. Additionally, the photodynamic activity of TMPyP was evaluated under these conditions at different irradiation times (Fig. 4b). The cell viability rapidly diminishes after only 5-min irradiation and the colony formation was not detected after 30 min.

A second methodology was evaluated, which involves the PDI of *C. albicans* cells that were not initially treated with

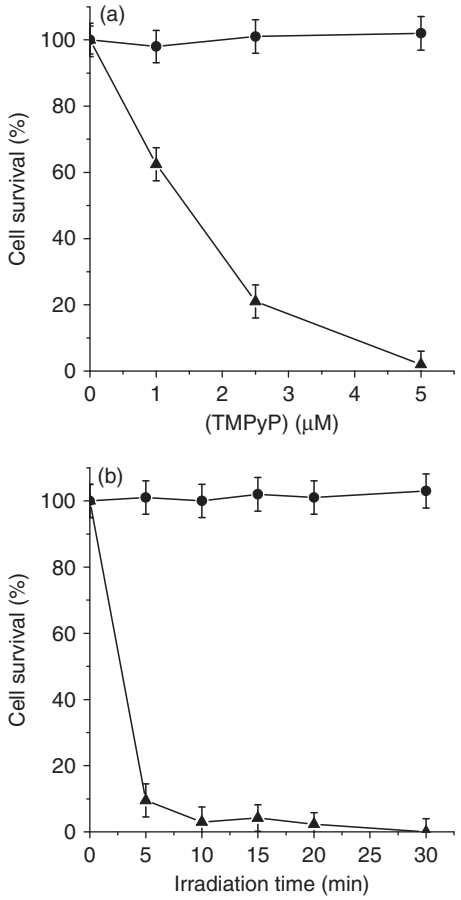

Fig. 4. Inactivation of *Candida albicans* cells on Sabouraud agar. The cell suspension (~10^6 CFU mL^{-1}) in PBS was incubated with TMPyP for 30 min at 37 °C in the dark, cells were plated on agar and irradiated with visible light. (a) Cells treated with different TMPyP concentrations (1, 5 and 10 μM) and irradiated for 30 min (▲). (b) cells incubated with 5 μM TMPyP and irradiated for different times (▲). Untreated control culture irradiated (●). Values represent mean ± SD of three separate experiments.

the sensitizer in solution. In these experiments, the cells were grown as a lawn on a Sabouraud agar surface with TMPyP impregnated in a small area. Different amounts of TMPyP (2.2, 4.5, 6.7 and 9.0 nmol) were homogeneously distributed (Fig. 5). The plates were spread with a suspension of *C. albicans*, which allows a lawn of yeast to be obtained. The cultures were kept in the dark for 30 min at 37 °C. The binding of TMPyP to cells occurred during this period. After that, the plates were irradiated with visible light for 30 min and incubated for 48 h at 37 °C in dark. Growth of *C. albicans* cells was not detected in the area treated with 6.7 and 9.0 nmol TMPyP (Fig. 5b). Similar behavior was found with 4.5 nmol; however, a smaller area of growth was found using 2.2 nmol. In contrast, modification of the lawn was not observed for controls treated with porphyrins and kept in the dark (Fig. 5a). Therefore, the photodynamic action induced by TMPyP led to cell death on the agar surface.

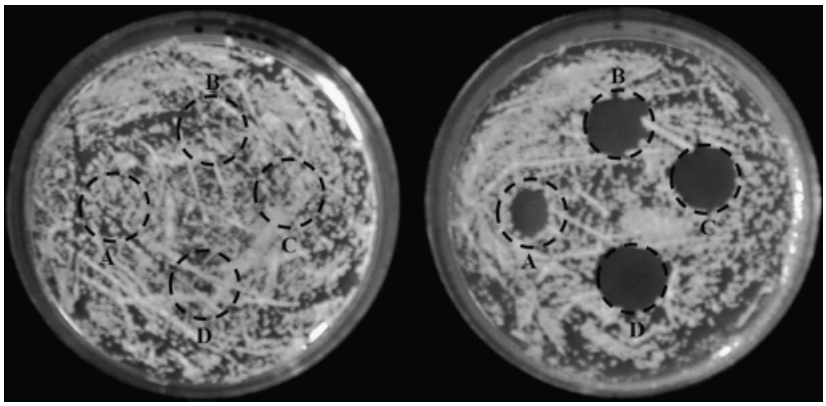

Fig. 5. Inactivation of *Candida albicans* cells on Sabouraud agar irradiated with visible light for 30 min. The dashed circles indicate the area where different amounts of sensitizer (A, 2.2; B, 4.5; C, 6.7 and D, 9.0 nmol) were spread from a solution of ~0.5 mM in water.

Photosensitization of *C. albicans* cells growing in colonies

The photodynamic activity of TMPyP was evaluated in colonies of *C. albicans* cells on Sabouraud agar. Thus, the appropriate dilution of *C. albicans* cellular suspensions in PBS was spread on Sabouraud agar plates to obtain small, separated colonies. The cultures were incubated overnight at 37 °C to form colonies of ~1 mm diameter. The colonies were treated with 9.0 nmol TMPyP, which was homogeneously distributed on the colony from a stock solution in water. After that, the plates were irradiated with visible light for 3 h and incubated at 37 °C in the dark. Characteristic results for TMPyP are shown in Fig. 6. The growth delay of *C. albicans* colonies on Sabouraud agar was clearly evident for colonies treated with TMPyP (Fig. 6a) with respect to the dark control or the control without sensitizer (Fig. 6b). A comparable increase in the colony area size was obtained in both control experiments. Thus, the colony area of the control increased approximately six times after one overnight incubation and 18 times in a second overnight incubation at 37 °C. In contrast, growth was not observed in colonies with PDI treatments even after 48-h incubation. Therefore, the growth delay observed after irradiation of the cultures treated with TMPyP is due to the photosensitization effect of the agent.

Comparable experiments were performed with colonies but exposing the culture dishes directly to midday sunlight (80 mW cm^{-2}) for 3 h (Fig. 7a). During the irradiation, the temperature of the cultures was about 35 °C. Thermal inactivation of the yeast did not occur, as also shown by control experiments (Fig. 7b). Under these conditions, a photodynamic action was observed similar to that found using light from a projector.

In both cases, growth of the colonies was not detected even 6 days post PDI treatment. To confirm the results, samples of these colonies treated with sensitizer and irradiated were aseptically transferred to fresh Sabouraud broth

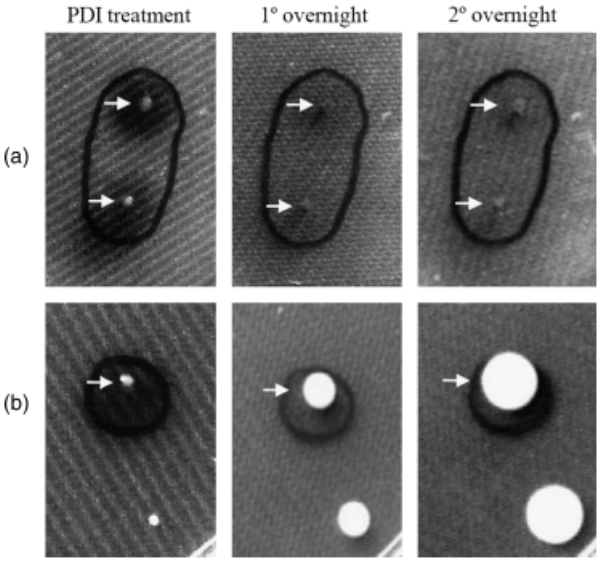

Fig. 6. Growth of *Candida albicans* colonies on Sabouraud agar treated with 9.0 nmol TMPyP. Sensitizer was spread on the colony (arrow) from a stock solution (~0.5 mM) in water and the culture incubated for 30 min at 37 °C before irradiation. (a) Colonies irradiated with visible light (90 mW cm^{-2}) for 3 h and incubated in the dark for one and two nights. (b) Control colonies were incubated at 37 °C in the dark.

medium and then to a new Sabouraud agar plate. After additional overnight incubation at 37 °C, no formation of colonies of *C. albicans* cells was detected, indicating a complete inactivation of yeast cells.

Discussion

The tetracationic porphyrin TMPyP is one of the most active water-soluble photosensitizers (Merchat *et al*., 1996a). The absorption spectrum of TMPyP is characterized by a typical *Soret* band at 424 nm and four *Q*-bands (520, 558, 584 and 638 nm), typical of free-base tetraphenylporphyrin derivatives (Kalyanasundaram & Neumann-Spallart,

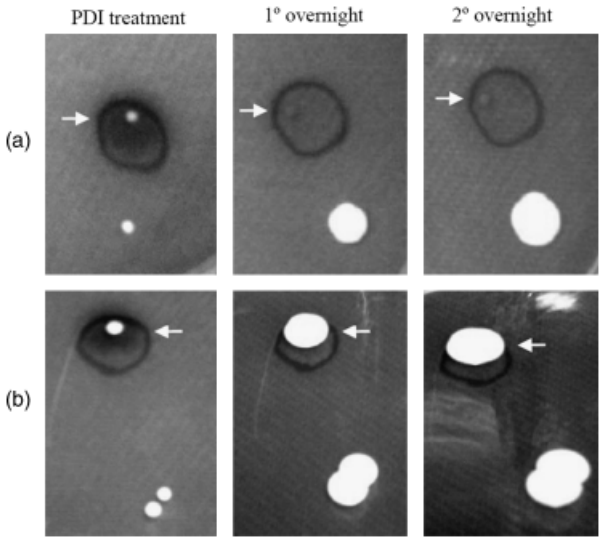

Fig. 7. Growth of *Candida albicans* colonies on Sabouraud agar treated with 9.0 nmol TMPyP. Sensitizer was spread on the colony (arrow) from a stock solution (~0.5 mM) in water and the culture incubated for 30 min at 37 °C before irradiation. (a) Colonies irradiated with natural sunlight at midday (80 mW cm^{-2}) for 3 h and incubated in the dark for one and two nights. (b) Control colonies incubated at 37 °C in the dark.

1982). The fluorescence emission spectrum shows two bands at 675 and 706 nm, with a fluorescence quantum yield (ϕ_F) of 0.011. Moreover, this porphyrin is highly efficient, with a quantum yield of $O_2(^1\Delta_g)$ production (Φ_Δ) of 0.74 in water (Praseuth *et al.*, 1986). This value is quite similar to that reported for TMAP ($\Phi_\Delta = 0.77$) in water (Verlhac *et al.*, 1984). However, the values of Φ_Δ can significantly change in a different medium, diminishing mainly when the sensitizer is partially aggregated (Milanesio *et al.*, 2008). Also, the photophysics of these porphyrins can be modified in the cellular microenvironment depending on where the sensitizer is located.

The binding of TMPyP to cells indicated that this porphyrin has a particularly high binding affinity for *C. albicans*. After different periods of incubation, the tendency observed for TMPyP was very similar to that found for TMAP (Cormick *et al.*, 2009). However, a slightly higher value of affinity was achieved for TMPyP with respect to TMAP, which reached ~1.35 nmol 10^{-6} cells (Cormick *et al.*, 2009). A fraction of the cell-bound TMPyP was removed by repeated washings with PBS, suggesting that some porphyrin molecules were weakly bound to the cells (Fig. 1). Thus, 41% TMPyP of the initially bound porphyrin was retained after one washing step for cells incubated for 30 min in the dark. However, the amount of retained porphyrin was lower when the cells were treated for 15 min (33%). Therefore, it appears that the molecules of TMPyP were more tightly bound to the cells with longer incubation times. Similar behavior was observed after a second washing, although the effect on the amount of cell-bound porphyrin was much less pronounced. On the other hand, after 30-min incubation, a minor difference was observed for porphyrin recovery from cells washed two and four times. Therefore, the amount of TMPyP remaining at this stage can be considered to represent the fraction of tightly bound porphyrin. A similar behavior was previously found using Gram-positive and Gram-negative bacterial cells (Merchat *et al.*, 1996a). In these microorganisms as well, the amount of TMPyP recovered from cells was slightly higher than the amount of TMAP recovered.

Photoinactivation of *C. albicans* induced by TMPyP was analyzed using different porphyrin concentrations (Fig. 2a) after 30-min irradiation. Cells treated with 1 μM TMPyP sensitizer showed a photoinactivation of 99.9% (~3 log decrease). No colony formation was detected when a higher porphyrin concentration (> 5 μM) was used, representing a cellular inactivation of > 99.999%.

The photodynamic action was also evaluated for different irradiation periods (Fig. 2b). A short irradiation time (5 min) produced ~99% photoinactivation of *C. albicans*. Under these conditions, an enhancement in the cell inactivation was found when the irradiation times were increased. A 30-min exposure to visible light was required to produce a photoinactivation of 5 log (Fig. 2b).

Comparable results were previously reported for *C. albicans* cells treated with TMAP (Cormick *et al.*, 2009). However, unlike TMPyP, the photoinactivation of yeast cells induced by TMAP was almost proportional to the amount of sensitizer in solution at this range of concentrations. This may indicate that a nonsaturation of the intracellular concentration of sensitizer occurred under these experimental conditions. Also, a short irradiation time (5 min) was sufficient to produce a high photoinactivation (~99.99%) of *C. albicans* cells treated with 5 μM of TMAP. Therefore, under these conditions, TMAP appears to be slightly more efficient than TMPyP at inactivating *C. albicans*. This behavior was also observed with a Gram-positive bacterium *Enterococcus seriolicida*, although the PDI was quite similar on a Gram-negative bacterium *Escherichia coli* (Merchat *et al.*, 1996a) using 10 μg L^{-1} of sensitizer. It is also clear that no difference between TMPyP and TMAP was found in *C. albicans* using 5 μM sensitizer and 30 min of irradiation; however, the efficiency of TMPyP was inferior to TMAP at a lower concentration or with a shorter irradiation period.

The effectiveness of TMPyP photoinactivation of *C. albicans* cells was evaluated after one washing step (Fig. 2b). Under these conditions, the photodynamic effect is mainly associated with porphyrin that is more tightly bound to cells. The photocytotoxic effect for cultures treated with 5 μM TMPyP diminished ~1 log with respect to unwashed cells, which is in agreement with a loss of the amount of cell-bound sensitizer after one washing step.

Only minor differences in the photoinactivation of *C. albicans* should be expected with successive washing steps.

The photodynamic action of TMPyP was analyzed on growth curves of *C. albicans*. These experiments were performed to ensure that PDI of cells is still possible when the cultures are not under starvation conditions or subject to the potentially damaging effects of phosphate buffer washing. In the presence of 5 μM TMPyP, the growth of *C. albicans* was arrested, although the photocytotoxic activity induced by this porphyrin is not enough to completely stop progress. Under these conditions, the effect of TMAP was also more effective than that of TMPyP to delay *C. albicans* growth (Cormick *et al.*, 2009).

On the other hand, photosensitized inactivation of *C. albicans* on surfaces can be used to inactivate cells growing *in vivo* as localized foci of infection, on skin or on an accessible area to be irradiated with either artificial visible light or natural sunlight (Orenstein *et al.*, 1998; Wood *et al.*, 1999). Also, photodynamic treatment was proposed as a new possibility for protecting food from microbial spoilage (Kreitner *et al.*, 2001). A high photoinactivation of *C. albicans* cells was found in cells treated with TMPyP in solution and irradiated on Sabouraud agar (Fig. 4). After 30-min irradiation, no colony formation was detected in *C. albicans* incubated with 5 μM TMPyP, in agreement with a high amount of cell-bound porphyrin in PBS suspension. Also, the PDI capacity of TMPyP remained high for immobilized cells on agar surfaces containing an area spread with different amounts of sensitizer (Fig. 5). Therefore, this porphyrin could be used to prevent yeast growth on surfaces that must be kept sterile, such as antimicrobial areas for surgery.

Growth of *C. albicans* cells as a colony led to an increase in resistance to antifungal PDI treatment compared with the resistance of cultures grown in suspension in liquid media. When the photoinactivation activities of these sensitizers were analyzed in *C. albicans* cells growing in colonies immobilized on Sabouraud agar surfaces, experiments with small colonies showed that the growth of the yeast was suppressed and a complete eradication of *C. albicans* cells was possible using 9 nmol TMPyP and 3 h of irradiation (Fig. 6). The photocytotoxic capacity of TMPyP remains high even for colonies irradiated with natural sunlight (Fig. 7). As compared with TMAP, both sensitizers produce a similar photosensitization effect and the increase in the colony size was similarly suppressed (Cormick *et al.*, 2009). These results indicate a highly efficient eradication of colonies of cells treated with TMPyP or TMAP porphyrins. As can be observed, the colony does not show evidence of growth and no viable cells remain after the PDI procedure. Therefore, the results indicated that under these experimental conditions, *C. albicans* cells growing in small colonies can be efficiently controlled by PDI treatment with TMPyP. This procedure could be used to treat localized infections by local delivery of the sensitizer into the infected area, using methods such as topical application (Dai *et al.*, 2009).

In summary, the present investigation indicates that TMPyP is an interesting sensitizer for application in PDI of *C. albicans* cells growing in cellular suspensions and as localized foci of infections.

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References

Ben Amor T & Jori G (2000) Sunlight-activated insecticides: historical background and mechanisms of phototoxic activity. *Insect Biochem Molec* **30**: 915–925.

Bliss JM, Bigelow CE, Foster TH & Haidaris CG (2004) Susceptibility of *Candida* species to photodynamic effects of photofrin. *Antimicrob Agents Ch* **48**: 2000–2006.

Brion LP, Uko SE & Goldman DL (2007) Risk of resistance associated with fluconazole prophylaxis: systematic review. *J Infection* **54**: 521–529.

Calzavara-Pinton PG, Venturini M & Sala R (2005) A comprehensive overview of photodynamic therapy in the treatment of superficial fungal infections of the skin. *J Photoch Photobio B* **78**: 1–6.

Carvalho GG, Felipe MP & Costa MS (2009) The photodynamic effect of methylene blue and toluidine blue on *Candida albicans* is dependent on medium conditions. *J Microbiol* **47**: 619–623.

Cormick MP, Alvarez MG, Rovera M & Durantini EN (2009) Photodynamic inactivation of *Candida albicans* sensitized by tri- and tetra-cationic porphyrin derivatives. *Eur J Med Chem* **44**: 1592–1599.

Dai T, Huang Y-Y & Hamblin MR (2009) Photodynamic therapy for localized infections – State of the art. *Photodiagnosis Photodyn Ther* **6**: 170–188.

Durantini EN (2006) Photodynamic inactivation of bacteria. *Curr Bioact Comp* **2**: 127–142.

Fidel PL (2006) *Candida*–host interactions in HIV-disease: relationships in oropharyngeal candidiasis. *Adv Dent Res* **19**: 80–84.

Gasparetto A, Lapinski TF, Zamuner SR, Khouri S, Alves LP, Munin E & Salvador MJ (2010) Extracts from *Alternanthera maritima* as natural photosensitizers in photodynamic antimicrobial chemotherapy (PACT). *J Photoch Photobio B* **99**: 15–20.

Goldman GH, da Silva Ferreira ME, dos Reis Marques E, Savoldi M, Perlin D, Park S, Godoy Martinez PC, Goldman MHS & Colombo AL (2004) Evaluation of fluconazole resistance mechanisms in *Candida albicans* clinical isolates from HIV-infected patients in Brazil. *Diagn Micr Infec Dis* **50**: 25–32.

Hamblin MR & Hasan T (2004) Photodynamic therapy: a new antimicrobial approach to infectious disease? *Photochem Photobio S* **3**: 436–450.

Jori G & Brown SB (2004) Photosensitized inactivation of microorganisms. *Photochem Photobio S* **5**: 403–405.

Kalyanasundaram K & Neumann-Spallart M (1982) Photophysical and redox properties of water-soluble porphyrins in aqueous media. *J Phys Chem* **86**: 5163–5169.

Kreitner M, Wagner K-H, Alth G, Ebermann R, Foißy H & Elmadfa I (2001) Haematoporphyrin- and sodium chlorophyllin-induced phototoxicity towards bacteria and yeasts – a new approach for safe foods. *Food Control* **12**: 529–533.

Kumar S & Kimball AB (2009) New antifungal therapies for the treatment of onychomycosis. *Expert Opin Inv Drug* **18**: 727–734.

Lambrechts SAG, Aalders MCG & Van Marle J (2005a) Mechanistic study of the photodynamic inactivation of *Candida albicans* by a cationic porphyrin. *Antimicrob Agents Ch* **49**: 2026–2034.

Lambrechts SAG, Aalders MCG, Verbraak FD, Lagerberg JWM, Dankert JB & Schuitmaker JJ (2005b) Effect of albumin on the photodynamic inactivation of microorganisms by a cationic porphyrin. *J Photoch Photobio B* **79**: 51–57.

Merchat M, Bertoloni G, Giacomini P, Villanueva A & Jori G (1996a) *Meso*-substituted cationic porphyrins as efficient photosensitizers of Gram-positive and Gram-negative bacteria. *J Photoch Photobio B* **32**: 153–157.

Merchat M, Spikes G, Bertoloni G & Jori J (1996b) Studies on the mechanism of bacteria photosensitization by *meso*-substituted cationic porphyrins. *J Photoch Photobio B* **35**: 149–157.

Milanesio ME, Alvarez MG, Bertolotti SG & Durantini EN (2008) Photophysical characterization and photodynamic activity of metallo 5-(4-(trimethylammonium)phenyl)-10,15,20-tris(2,4,6-trimethoxyphenyl)porphyrin in homogeneous and biomimetic media. *Photochem Photobio S* **7**: 963–972.

Munin E, Giroldo LG, Alves LP & Costa MS (2007) Study of germ tube formation by *Candida albicans* after photodynamic antimicrobial chemotherapy (PACT). *J Photoch Photobio B* **88**: 16–20.

Orenstein A, Klein D, Kopolovic J, Winkler E, Malik Z, Keller N & Nitzan Y (1998) The use of porphyrins for eradication of *Staphylococcus aureus* in burn wound infections. *FEMS Immunol Med Mic* **19**: 307–314.

Osumi M (1998) The ultrastructure of yeast: cell wall structure and formation. *Micron* **29**: 207–233.

Praseuth D, Gaudemer A, Verlhac J-B, Kraljic I, Sissoëff I & Guillé E (1986) Photocleavage of DNA in the presence of synthetic water-soluble porphyrin. *Photochem Photobiol* **44**: 717–724.

Ribeiro MA & Rodrigues CP (2007) Up-regulation of ERG11 gene among fluconazole-resistant *Candida albicans* generated *in vitro*: is there any clinical implication? *Diagn Micr Infec Dis* **57**: 71–75.

Salmon-Divon M, Nitzan Y & Malik Z (2004) Mechanistic aspect of *Escherichia coli* photodynamic inactivation by cationic tetra-meso(*N*-methylpyridyl)porphine. *Photochem Photobio S* **3**: 423–429.

Valduga G, Breda B, Giacometti GM, Jori G & Reddi E (1999) Photosensitization of wild and mutant strains of *Escherichia coli* by *meso*-tetra(*N*-methyl-4-pyridyl)porphine. *Biochem Bioph Res Co* **256**: 84–88.

Verlhac J-B, Gaudemer A & Kraljic I (1984) Water soluble porphyrins and metallo-porphyrins as photosensitizers in aerated aqueous solutions. I. Detection and determination of quantum yield of formation of singlet oxygen. *Nouv J Chim* **8**: 401–406.

Wainwright M (2004) Photoinactivation of viruses. *Photochem Photobio S* **5**: 406–411.

Wood S, Nattress B, Kirkham J, Shore R, Brookes S, Griffiths J & Robinson C (1999) An *in vitro* study of the use of photodynamic therapy for the treatment of natural oral plaque biofilms formed *in vivo*. *J Photoch Photobio B* **50**: 1–7.