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Photodynamic inactivation of *Candida albicans* by a tetracationic tentacle porphyrin and its analogue without intrinsic charges in presence of fluconazole

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ABSTRACT

The photodynamic inactivation mediated by 5,10,15,20-tetrakis[4-(3-*N*,*N*-dimethylaminopropoxy)phenyl]porphyrin (TAPP) and 5,10,15,20-tetrakis[4-(3-*N*,*N*,*N*-trimethylaminepropoxy)phenyl]porphyrin ($TAPP^{4+}$) were compared in *Candida albicans* cells. A strong binding affinity was found between these porphyrins and the yeast cells. Photosensitized inactivation of *C. albicans* increased with both photosensitizer concentration and irradiation time. After 30 min irradiation, a high photoinactivation (~5 log) was found for *C. albicans* treated with 5 μM porphyrin. Also, the photoinactivation of yeast cells was still elevated after two washing steps. However, the photocytotoxicity decreases with an increase in the cell density from 10^6 to 10^8 cells/mL. The high photodynamic activity of these porphyrins was also established by growth delay experiments. This *C. albicans* strain was susceptible to fluconazole with a MIC of 1.0 μg/mL. The effect of photosensitization and the action of fluconazole were combined to eradicate *C. albicans*. After a PDI treatment with 1 μM porphyrin and 30 min irradiation, the value of MIC decreased to 0.25 μg/mL. In addition, a complete arrest in cell growth was found by combining both effects. TAPP was similarly effective to photoinactivate *C. albicans* than $TAPP^{4+}$. This porphyrin without intrinsic positive charges contains basic amino groups, which can be protonated at physiological pH. Moreover, an enhancement in the antifungal action was found using both therapies because lower doses of the agents were required to achieve cell death.

1. Introduction

Fungal infections have increased significantly in recent years. Now they represent an exponentially growing threat for human health due to a combination of difficult diagnosis and a shortage of effective antifungal drugs [1]. Candidiasis is an opportunist fungal infection usually caused by *Candida albicans*. This dimorphic yeast is naturally found in most humans among the normal microbiota of the skin, mucous membranes in the oral cavity, bowel and in the urogenital tract of females [2]. Because of the common eukaryotic nature of fungal and human cells, it is difficult to identify specific metabolic or structural antimicrobial targets for fungi [3]. The fundamental physiological role and the different composition of cholesterol in humans and ergosterol in fungi render the cytoplasmic membrane of *Candida* a suitable target for the action of antifungals. Triazoles are inhibitors of the cytochrome P450 14α-sterol demethylase (CYP51), an essential enzyme in the biosynthesis of ergosterol. The current treatment for candidiasis heavily relies on triazoles, even though patient responses to these antifungal drugs tend to be slow with a high risk of reinfection. Especially fluconazole has been extensively used in clinical practice due to its great efficacy and reduced toxicity. However, antifungal-resistant *C. albicans* strains have emerged with frequent exposure to fluconazole [1]. Moreover, the development of new agents in clinical therapy has lagged behind due to increasing incidence of drug resistance and few effective antifungal agents are available. Also, inadequate dosing may contribute to treatment failure and the emergence of resistance [4]. Thus, new therapies are being searched for treating fungal infections. An interesting alternative is represented by photodynamic inactivation (PDI) of microorganisms [5,6]. PDI involves the addition of a phototherapeutic agent, which is rapidly bound to cells. The aerobic irradiation of the infection with visible light produces highly reactive oxygen species (ROS), which rapidly react with a variety of substrates inducing

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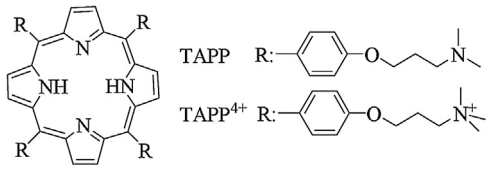

Scheme 1. Structure of the TAPP and TAPP^{4+} porphyrins.

damage in biomolecules. These reactions induce a loss of biological functionality leading to cell inactivation [7]. Thus, experimental investigations have demonstrated that yeasts can be effectively photoinactivated *in vitro* by several photosensitizers [8].

In recent years, efforts have been made to overcome the emergence of resistant fungi by using drug combinations [9]. Various approaches have been proposed to increase the susceptibility of *C. albicans* to fluconazole in order to cope with treatment failures. An advantage of using combinations is the additive effect, in which antifungal activity is greater than the individual contribution of each agent. Benefits can include a wider spectrum of efficacy, improved safety and a reduction in antifungal resistance. Thus, it is possible to use PDI in combination with other therapeutic approaches.

In the present work, we investigated the susceptibility of *C. albicans* to the phototoxic effect produced by 5,10,15,20-tetrakis [4-(3-*N,N*-dimethylaminopropoxy) phenyl]porphyrin (TAPP) and 5,10,15,20-tetrakis [4-(3- *N,N,N*-trimethylaminepropoxy) phenyl]porphyrin (TAPP^{4+}) (Scheme 1). It was previously demonstrated that TAPP^{4+} is an active photosensitizer to inactivate *Escherichia coli* cells [10]. Also, we studied spectroscopic and photodynamic properties of both porphyrins in different media [11]. The photooxidation of substrates photosensitized by TAPP indicated that this intrinsically non-charged porphyrin can be an interesting agent for the photodynamic inactivation of microorganisms. Therefore, the photoinactivation activity of TAPP and TAPP^{4+} were compared to establish conditions for the eradication of *C. albicans* in cell suspensions. Then, PDI mediated by these porphyrins was combined with an antifungal therapy using fluconazole to enhance both approaches.

2. Materials and methods

2.1. General

Absorption spectra were recorded on a Shimadzu UV-2401PC spectrometer (Shimadzu Corporation, Tokyo, Japan). Fluorescence measurements were performed on a Spex FluoroMax spectrofluorometer (Horiba Jobin Yvon Inc., Edison, NJ, USA). Quartz cells of 1 cm path length were used at room temperature. The cell suspensions were irradiated with a Novamat 130 AF slide projector (Braun Photo Technik, Nürnberg, Germany) equipped with a 150 W lamp. A wavelength range between 350 and 800 nm was selected by optical filters. The light was filtered through a 2.5 cm glass cuvette filled with water to absorb heat. Experiments were performed at room temperature with a fluence rate of 90 mW/cm^2 (Radiometer Laser Mate-Q, Coherent, Santa Clara, CA, USA). Cell growth was measured with a Turner SP-830 spectrophotometer (Dubuque, IA, USA). Chemicals from Aldrich (Milwaukee, WI, USA) were used without further purification. Fluconazole was purchased from Sigma (St. Louis, MO, USA).

2.2. Photosensitizers

5,10,15,20-Tetrakis[4-(3-*N,N*-dimethylaminopropoxy)phenyl]-porphyrin (TAPP) and 5,10,15,20-tetrakis[4-(3-*N,N,N*-trimethylaminepropoxy)phenyl]porphyrin (TAPP^{4+}) were synthetized as previously described [12]. Stock solutions (0.5 mM) of TAPP and TAPP^{4+} were prepared by dissolution in 1 mL of *N,N*-dimethylformamide (DMF). The concentration was checked by spectroscopy, taking into account the value of molar absorptivity [12].

2.3. C. albicans *culture conditions*

The strain of *C. albicans* PC31, recovered from human skin lesion, was previously characterized and identified [13]. Yeast was grown aerobically overnight in Sabouraud (Britania, Buenos Aires, Argentina) broth (SB, 3% w/w, 4 mL) at 37 °C to stationary phase. Cells were harvested by centrifugation of broth cultures (3000 rpm for 15 min) and re-suspended in 4 mL of 10 mM phosphate-buffered saline (PBS, pH 7.0), corresponding to ~10^7 colony forming units (CFU)/mL. The cells were appropriately diluted to obtain ~10^6 CFU mL^{-1} or concentrated to get ~10^8 CFU/mL in PBS. In all the experiments, 2 mL of the cell suspensions in Pyrex brand culture tubes (13 × 100 mm) were used and the photosensitizer was added from the corresponding stock solution. Cell suspensions were serially diluted with PBS and each solution was quantified by using the spread plate technique in triplicate. Viable *C. albicans* cells were monitored and the number of CFU was determined on Sabouraud agar (SA) plates after ~48 h incubation at 37 °C.

2.4. *Porphyrin binding to* C. albicans *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 and 5 μM) of TAPP and TAPP^{4+} for different times. The cultures were centrifuged (3000 rpm for 15 min) and resuspended with PBS. The cell pellets, after different washing steps (0 and 2) obtained by centrifugation (3000 rpm for 15 min) were resuspended in 2% aqueous sodium dodecyl sulfate (SDS) 2 mL. After that, the cells was incubated overnight at 4 °C and sonicated for 30 min. The concentration of porphyrin in the solution was measured by spectrofluorimetry (TAPP λ_{exc} = 422 nm, λ_{em} = 659 nm; TAPP^{4+} λ_{exc} = 420 nm, λ_{em} = 660 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 concentrations of the porphyrins in the samples were determined from a calibration curve obtained with standard solutions of the each porphyrin in 2% SDS ([porphyrin] ~0.005–0.1 μM).

2.5. *Photosensitized inactivation of* C. albicans *cells*

Cell suspensions of *C. albicans* (2 mL in Pyrex culture tubes of 13 × 100 mm, ~10^6 CFU mL^{-1}) in PBS were incubated with an appropriate concentration (1 and 5 μM) of porphyrin for 30 min in the dark at 37 °C. After that, the culture tubes were exposed to visible light from the bottom for different time intervals [14]. It was also studied the strength binding of the porphyrins by the cells, performing one or two washes of the suspensions, after of added the porphyrin and before illumination. Cell viability was checked as described above.

2.6. *Growth curves 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 3% w/w fresh SB medium. The suspension was homogenized and aliquots of 2 mL were incubated with 5 μM of sensitizer at 37 °C. The flasks were then irradiated with visible light at 37 °C. *C. albicans* growth was followed spectroscopically at 650 nm.