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Application of benzo[*a*]phenoxazinium chlorides in antimicrobial photodynamic therapy of *Candida albicans* biofilms

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ABSTRACT

The use of Antimicrobial Photodynamic Therapy (APDT) as a new approach to treat localized *Candida* infections is an emerging and promising field nowadays. The aim of this study was to verify the efficacy of photodynamic therapy using two new benzo[*a*]phenoxazinium photosensitizers against *Candida albicans* biofilms: *N*-(5-(3-hydroxypropylamino)-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene)ethanaminium chloride (**FSc**) and *N*-(5-(11-hydroxyundecylamino)-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene)ethanaminium chloride (**FSd**). The photodynamic activity of dyes against *C. albicans* biofilms was evaluated by incubating biofilms with dyes in the range of 100–300 μM for 3 or 18 h followed by illumination at 12 or 36 J cm^{-2}, using a xenon arc lamp (600 ± 2 nm). A total photoinactivation of *C. albicans* biofilm cells was achieved using 300 μM of **FSc** with 18 h of incubation, followed by illumination at 36 J cm^{-2}. Contrarily, **FSd** had insignificant effect on biofilms inactivation by APDT. The higher uptake of **FSc** than **FSd** dye by biofilms during the dark incubation may explain the greater photodynamic effectiveness achieved with **FSc**. The results obtained stresses out the **FSc**-mediated APDT potential use to treat *C. albicans* infections.

1. Introduction

In the last decades, the incidence of superficial and systemic fungal infections has increased due to several factors, including the more frequent use of invasive procedures, prosthetic devices, immunosupressive medication and broad-spectrum antibiotics, as well as the increased incidence of neutropenia and HIV infections [1]. *Candida albicans* is the most virulent *Candida* species and represents an important public health challenge with a high economic and medical relevance due to the increased costs of care, time of hospitalization and high morbidity and mortality rates, especially on immunocompromised patients [2]. The pathogenicity of *C. albicans* may be attributed to several virulence factors, including production of some tissue-damaging hydrolytic enzymes, as well as adherence and biofilm formation on host tissues and medical devices (e.g. catheters and prosthetic devices) [2]. Biofilm formation is one of the main virulence factors of *C. albicans*, because these communities show different phenotype characteristics than their planktonic counterparts, particularly increased resistance to antimicrobial agents [3,4]. The clinical significance of biofilms is highlighted by recent estimates that over 65% of all hospital infections are originated by these microbial communities [5,6]. The use of conventional antifungal treatments can be prolonged, expensive and has been more and more associated with the appearance of resistant strains in the last few years. For this reason, there is an increased need of more effective and localized antifungal therapeutic options to treat fungal infections [7]. Photodynamic therapy, originally developed for the treatment of skin tumors, has been shown as an effective therapy to eliminate bacteria and fungi that cause localized infections of the skin and oral cavity [8–21]. Antimicrobial Photodynamic Therapy (APDT) combines a non-toxic and light sensitive dye, termed photosensitizer, with oxygen and harmless visible light with appropriate wavelength. Upon illumination, the photosensitizer is excited and can undergo reaction with ambient oxygen resulting in the formation of Reactive Oxygen Species (ROS), leading to oxidative stress that can cause growth inhibition and cell death [22]. As photosensitizers may cross the plasma membrane and take different subcellular localizations, several intracellular components may be affected during the oxidative stress caused by ROS produced, affecting the cell functions and its

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metabolism in a manner that can lead to apoptosis [21]. These include plasma membrane, mitochondria, vacuoles, Golgi apparatus and endoplasmic reticulum [23]. The main ROS produced upon light excitation is the termed singlet oxygen ($^1O_2$), a form of oxygen that is very short lived, extremely reactive and has a very limited diffusivity (in a radius of approximately 20 nm) [22]. For these reasons this therapy can have a simultaneous localized effect, where only molecules and structures within the microenvironment of the photosensitizer are affected by singlet oxygen [22,23]. Thus, sterilization of oral cavities and root canals, as well as treatment of localized infections seems to be the main promising applications of APDT [20,21,24,25].

Several forms of photosensitizers have been created during the last few years, particularly dyes belonging to the class of porphyrins, phthalocyanines and phenothiazines [7,25,26]. Cationic photosensitizers with an absorption peak wavelength in the far red (600–800 nm) seem to be the more effective structures to kill both bacteria and fungi [7,22,25]. Accordingly to Foley et al. [27], some benzo[*a*]phenoxazinium chalcogen analogues can have great photoinduced activity against yeasts and bacteria, such as *C. albicans*, *Escherichia coli* and *Enterococcus faecalis* [26]. In this context, the main objective of the present work was to investigate the potential antifungal photodynamic activity using two new benzo[*a*]phenoxazinium chlorides against *C. albicans* biofilms.

2. Materials and methods

2.1. Organisms and growth conditions

The strain *C. albicans* ATCC 90028, was preserved at −80 °C in a storage solution of Sabouraud Dextrose Broth (SDB; Liofilchem®, Roseto degli Abruzzi, Italy) supplemented with 20% glycerol. Prior to each experiment, yeast cells were sub-cultured on Sabouraud Dextrose Agar (SDA; Liofilchem®, Roseto degli Abruzzi, Italy) for 48 h at 37 °C. Then, one loopful of single-cell colonies (3–5 colonies) was inoculated in 30 ml of SDB and incubated for 18 h, at 37 °C, under constant agitation at 120 rev/min. After this step, cells were harvested by centrifugation at 3000*g* for 10 min at 4 °C and washed twice with 15 ml of Phosphate Buffer Solution (PBS – pH 7.5; 0.1 M). The pellets were then suspended in 10 ml of SDB and the cellular density was adjusted to 1×10^7 cells ml^{-1} using an improved Neubauer haemocytometer (Marienfild, Land-Könicshofen, Germany). Biofilms were grown at 37 °C under agitation at 120 rev/min for 48 h, in a 96-wells polystyrene microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium), with the reposition of 100 µl of fresh medium after 24 h. In each plate, an interval of three wells between each well was used to avoid side light exposure during biofilms photoinactivation. After biofilm formation the medium was aspirated and non-adherent cells removed by washing biofilms once with PBS.

2.2. Photosensitizers

The benzo[*a*]phenoxazinium chlorides termed *N*-(5-(3-hydroxypropylamino)-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene)ethanaminium chloride – **FSc** and *N*-(5-(11-hydroxyundecylamino)-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene)ethanaminium chloride – **FSd** (Fig. 1) were previously synthetized in the Department of Chemistry of University of Minho [28,29] and the respective powders were solubilized in Dimethyl Sulfoxide (DMSO) to a final concentration of 10 mM. Then, the solutions were transferred to sterile amber microtubes (VWR International, LLC. USA) to avoid ambient light and were stored at 4 °C. Immediately before each experiment, aliquots of the dyes were diluted in sterile PBS to obtain working solutions of 100, 200 and 300 µM.

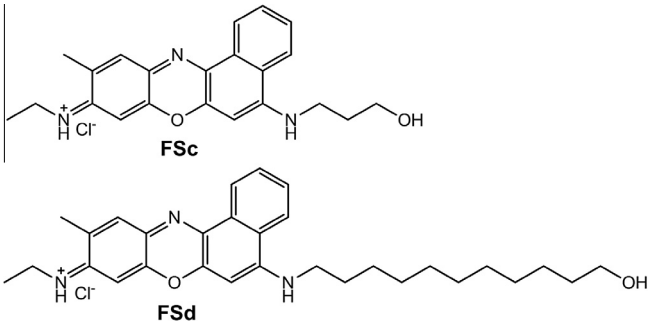

Fig. 1. Structures of benzo[*a*]phenoxazinium chlorides **FSc** and **FSd**.

Table 1
Photophysical properties of dyes in ethanol, DMSO and PBS, namely, wavelength of maximum absorption (λ_{max}), excitation wavelength (λ_{ex}), emission wavelength (λ_{em}) and relative fluorescence quantum yield (Φ_F).

Dye	Solvent	λ_{max} (nm)	λ_{em} (nm)	Φ_F
FSc	Ethanol	628	646	0.41
	DMSO	631		
	PBS	618/579		
FSd	Ethanol	631	654	0.29
	DMSO	639		
	PBS	615		

Table 1 shows the main photophysical properties of both dyes. The absorption spectra in ethanol, PBS buffer and DMSO are shown in Fig. 2. The compound with a longer alkyl chain (**FSd**) is slightly red-shifted showing a higher fraction of a band near 500 nm, that corresponds to the neutral basic form [28,29]. Also, as expected, compound **FSd** has much less solubility in PBS aqueous buffer. In biofilm compound **FSd** is probably in a less hydrated environment than compound **FSc**. Considering the higher tendency of **FSd** to deprotonate, the absorption at the irradiation wavelength is expected to be lower than for **FSc**.

2.3. Photodynamic inactivation

After the biofilms were formed, dyes (200 µl) were added to biofilms and the plates were incubated in the dark during 3 or 18 h at 37 °C under agitation (120 rev/min). Dyes were then aspired and biofilms were washed twice with PBS to remove loosely attached cells and excess of dye. After, 200 µl of PBS were

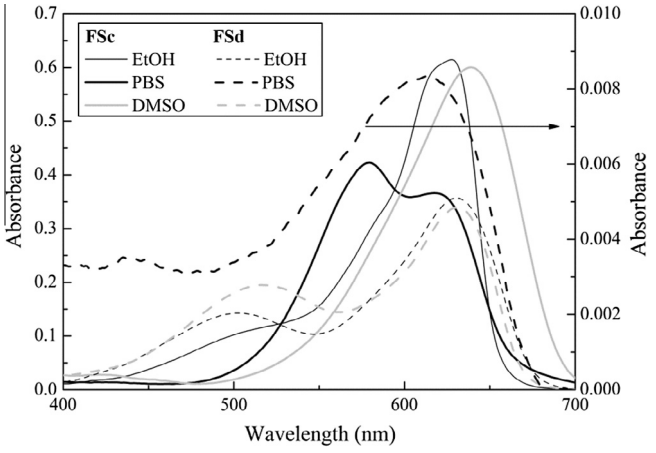

Fig. 2. Absorption spectra of compounds **FSc** and **FSd** in ethanol (EtOH), PBS and DMSO.