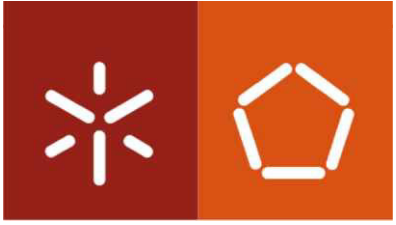

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Dissertação de Mestrado
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Trabalho realizado sob a orientação da
Professora Doutora Mariana Contente Rangel Henriques
Professora Doutora Isabel Maria Pires Belo

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ABSTRACT

In recent decades, the incidence of fungal infections caused by *Candida* spp. has significantly increased. *C. albicans* is the most important pathogen within this genus, since it is the most commonly isolated. However, other *Candida* species have gained importance over the last years, namely *C. glabrata* and *C. parapsilosis.* The rise of strains highly resistant to conventional antifungal treatments has increased the research for development of new effective antifungal therapies. Antimicrobial Photodynamic Therapy (APDT) has been proposed as a therapy for a large variety of localized infections.

The aim of this work was to study the antifungal photodynamic effect of a new benzophenoxazine compound – FSc – on *C. glabrata, C. parapsilosis* and *C. albicans* biofilms. Biofilms were treated with different FSc concentrations (50 μM – 500 μM) for 18 h and irradiated with a xenon arc lamp (600 ± 2 nm) at various light doses (8 J cm^{-2} – 50 J cm^{-2}). The efficiency of photodynamic treatment mediated by two widely used dyes – Nile blue chloride and Protoporphyrin IX – on *C. albicans* biofilms was also evaluated. Biofilms were incubated with 300 μM of each dye for 18 h and irradiated with a light dose of 36 J cm^{-2}. In order to understand the oxidative stress response of *Candida* species to APDT, suspensions of *C. albicans*, *C. glabrata* and *C. parapsilosis* were incubated with 2 mM and 20 mM of two oxidant agents, hydrogen peroxide and paraquat, for 3 h. The effect of these oxidants on both cell viability and induction of antioxidant enzymes was evaluated.

The photoinactivation of *Candida* biofilms with either FSc or PpIX as a photosensitizer was able to reduce the cell viability. On the other hand, Nile blue-mediated APDT assays showed no effect on cell viability. In general, photoinactivation of *Candida* cells was dependent on the dye concentration and light dose delivered. Nevertheless, irradiation with increasing light doses only resulted in a slight decrease of biofilm viability. Although the results of this study are encouraging, further investigations are needed to optimize protocols for maximal cell inactivation.

Candida species showed high levels of resistance to the two oxidant agents, with the exception of *C. glabrata* that presented a greater sensitivity to paraquat. Generally, all *Candida* strains responded to hydrogen peroxide and paraquat exposure by increasing their content of catalase and superoxide dismutase, respectively. In summary, the data suggest that *Candida* species have different levels of resistance to oxidant agents and they use different defense mechanisms against these substances, showing differences in the induction of antioxidant enzymes.

RESUMO

Nas últimas décadas, a incidência de infeções fúngicas provocadas por espécies do género *Candida* tem aumentado significativamente. *C. albicans* é considerada a espécie mais patogénica dentro deste género, uma vez que é a mais comummente isolada. No entanto, nos últimos anos, outras espécies de *Candida* têm ganho importância, nomeadamente *C. glabrata* e *C. parapsilosis*. O aumento de estirpes altamente resistentes aos tratamentos antifúngicos convencionais tem aumentado a necessidade de desenvolvimento de novas terapias antifúngicas eficazes. A Terapia Fotodinâmica Antimicrobiana (TFDA) tem sido proposta como um tratamento para uma grande variedade de infeções localizadas.

Este trabalho teve como objetivo estudar o efeito fotodinâmico de um corante pertencente ao grupo das benzofenoxazinas – FSc – em biofilmes de *C. glabrata, C. parapsilosis* e *C. albicans.* Os biofilmes foram incubados com diferentes concentrações de FSc (50 μM – 500 μM), durante 18 h, e irradiados com várias doses de luz (8 J cm^{-2} – 50 J cm^{-2}), usando uma lâmpada de arco de xénon (600 ± 2 nm). Foi ainda avaliada a eficiência da TFDA com dois corantes amplamente utilizados – Azul do nilo e Protoporfirina IX – em biofilmes de *C. albicans*. Estes foram incubados com 300 μM de cada corante, durante 18 h, e irradiados com uma dose de luz de 36 J cm^{-2}. De forma a compreender a resposta das espécies de *Candida* ao stresse oxidativo induzido pela TFDA, suspensões de *C. albicans*, *C. glabrata* e *C. parapsilosis* foram incubadas com 2 mM e 20 mM de dois agentes oxidantes, peróxido de hidrogénio e paraquato, durante 3 h. Determinou-se o efeito destes oxidantes na viabilidade celular e na indução de enzimas antioxidantes.

Ao contrário da TFDA com Azul do nilo, a fotoinativação de biofilmes de *Candida* com os compostos FSc ou PpIX foi capaz de reduzir a viabilidade celular. De um modo geral, a inativação das células de *Candida* foi dependente da concentração de corante e da dose de luz aplicada. No entanto, a irradiação com doses crescentes de luz resultou apenas numa ligeira diminuição da viabilidade celular. Embora os resultados deste estudo sejam encorajadores, investigações posteriores são necessárias para otimizar os protocolos de modo a induzir uma maior inativação celular.

As espécies de *Candida* apresentaram uma grande resistência aos dois agentes oxidantes, à exceção de *C. glabrata* que mostrou ser mais sensível ao paraquato. De um modo geral, as espécies de *Candida* responderam à exposição ao peróxido de hidrogénio e ao paraquato com a indução de catalase e de superóxido dismutase, respetivamente. Em suma, os resultados indicam que as espécies de *Candida* possuem distintos níveis de resistência aos agentes oxidantes, utilizando diferentes mecanismos de defesa contra esses mesmos compostos.

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LIST OF ABBREVIATIONS

ABC	ATP-binding Cassette
Abs	Absorbance
ALA	5-Aminolevulinic Acid
ANOVA	Analysis of Variance
APDT	Antimicrobial Photodynamic Therapy
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
CAT	Catalase
CFU	Colony Forming Units
CHROMagar	Chromogenic Media Agar
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EDTA	Ethylenediamine Tetraacetic Acid
g	Gravity constant (m s^{-2})
GNP	Gold Nanoparticles
HIV	Human Immunodeficiency Virus
HSP	Heat Shock Proteins
Log	Logarithm
M	Molarity (mol L^{-1}; mmol L^{-1}; µmol L^{-1})
MB	Methylene Blue
MFS	Major Facilitator Superfamily
MIC	Minimum Inhibitory Concentration
MOPS	3-(N-Morpholino)propanesulfonic acid, 4-Morpholinepropanesulfonic acid
NCAC	Non-*C. albicans Candida*
NMB	New Methylene Blue
p	Significance value
PBS	Phosphate Buffered Saline
PDT	Photodynamic Therapy

PpIX	Protoporphyrin IX
PQ	Paraquat
PS	Photosensitizer
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
SDA	Sabouraud Dextrose Agar
SDB	Sabouraud Dextrose Broth
SOD	Superoxide Dismutase
TB	Toluidine Blue
TBO	Ortho-toluidine Blue
%	Percentage

PREAMBLE

Over the last 30 years, the incidence of fungal infections in humans has significantly increased due to several factors, including the use of invasive procedures, prosthetic devices, application of immunosuppressive medications and broad-spectrum antibiotics, as well as the increased incidence of neutropenia and human immunodeficiency virus (HIV) infections [1, 2]. *Candida* species are among the major human fungal pathogens and have become the second leading cause of invasive fungal infections, being responsible for 70-90 % of all cases [3]. Moreover, systemic candidiasis was observed in 20-40 % of patients with cancer and in approximately 25 % of patients who received bone marrow transplants [4]. Most cases of candidiasis have been attributed to *C. albicans*, but recently, non-*C. albicans Candida* (NCAC) spp., such as *C. glabrata* and *C. parapsilosis*, have been identified as common pathogens [5]. The worldwide increase in antimicrobial resistance, mainly due to inappropriate prescription of antibiotics and mechanisms developed by microbial cells to increase their resistance to external insults [6] has resulted in the search for alternative antimicrobial therapies [7, 8].

Photodynamic therapy (PDT) initially developed to combat cancerous lesions, has been employed in the treatment of infectious diseases. In fact, it has been found an effective antimicrobial action of PDT against a wide variety of microorganisms, including bacteria and fungi, even in antibiotic resistant strains [9–11], viruses and protozoa [12–17]. Antimicrobial Photodynamic Therapy (APDT) employs a non-toxic dye, termed photosensitizer (PS), and low intensity visible light which, in the presence of oxygen, combine to produce cytotoxic oxygen species leading to cell death.

The development of new classes of PSs, the optimization of dosimetry and further enhancement in technology may drastically change the currently achieved APDT clinical outcome. Thus, this dissertation aimed to evaluate the inactivation of *C. glabrata, C. parapsilosis* and *C. albicans* biofilms by APDT through the use of a new benzophenoxazine compound. Dye concentration and light dose were optimized in order to improve the levels of cell inactivation. In addition, the antimicrobial activity of APDT mediated by two commonly used dyes, Nile blue chloride and Protoporphyrin IX, against *C. albicans* biofilms was also evaluated. With the intent to understand how cells respond to oxidative stress, the main cause of cell death during APDT, cell viability and antioxidant enzymes activity of the three *Candida* species after exposure to hydrogen peroxide and paraquat (oxidant agents) were

analyzed. It was also determined the minimum inhibitory concentration of both oxidant agents for the three *Candida* species under study.

The present dissertation is organized into four chapters. Chapter 1 is a brief review of the theoretical basis associated to this work, including cell biology of *Candida* spp. and their biofilm forming ability; the principles of APDT; the main PSs and light sources used; APDT applications and mechanisms of resistance; and the effect of oxidative stress on cells. Chapter 2 includes the materials and methods used in the experimental work. On chapter 3, all the results obtained, as well as its discussion are presented. Finally, chapter 4 highlights the main conclusions obtained from the realized work and presents some suggestions for future works.

1. CHAPTER I

INTRODUCTION

1.1. *Candida* species

The *Candida* genus comprises a heterogeneous group of 150 species [18] that are common commensal inhabitant on human mucosal surfaces and skin in an asymptomatic manner. Nevertheless, when that outer layer of protection is compromised, *Candida* spp. can cause local infection (candidosis). In immunocompromised individuals it can infect deeper layers and if it becomes systemic (candidemia), can lead to death [19, 20]. *Candida* spp. are most frequently isolated from the oral cavity, gastrointestinal and genital tracts, and can be detected in approximately 31 to 55 % of healthy individuals [21].

C. albicans is the most important pathogen within the *Candida* genus [22], representing over 80 % of all yeasts isolated in clinical samples [18]. This is the most common fungus affecting oral cavity and immunocompromised patients, namely HIV infected patients and those with oncologic malignancies [7]. However, there are many other *Candida* species that have gained importance over the last years, because they are also known to be etiological agents of human infections [5], including: *C. glabrata, C. parapsilosis, C. tropicalis, C. krusei, C. kefyr, C. rugosa and C. dubliniensis* [23]. *C. lusitanae, C. orthopsilosis, C. guilliermondii* and *C. metapsilosis* are also pathogenic species but less relevant in comparison with the previous ones [5]. The apparent increased occurrence of non-*Candida albicans Candida* (NCAC) spp. may be related with their inherent higher level of resistance to different antifungal agents compared to *C. albicans,* and the improvement of diagnostic methods [24].

Some of these species may co-exist competitively and symbiotically in dual biofilms. This is the case of *C. albicans* and *C. glabrata* [25] that are the most frequent combination between *Candida* spp. and have been found in approximately 70 % of the patients with oral candidosis [26]. In addition to the epithelial and mucosal surfaces, *Candida* can colonize numerous medical devices (vascular catheter, joint prosthesis, hemodialysis fistulas, and peritoneal dialysis catheters).

1.1.1. Cell Biology

Candida species are taxonomically classified in the Ascomycota phylum, and are formed by structures called blastoconidia, which may be round or elongated. They are surrounded by a rigid cell wall that is composed of a mixture of glucan, mannan, chitin and lipoproteins, separated from

the plasma membrane by a periplasmic space [2]. Moreover, some *Candida* species may also produce a filamentous type of growth such as true hyphae or more frequently, pseudohyphae. Pseudohyphae are formed when the production of blastoconidia continues without separation of the conidia from each other, resulting in filaments with constrictions at the cell-cell junctions. There is no internal cross walls associated with this growth form. In comparison, true hyphae result of a 'germ tube' projection which can elongate and then branch with defined septa that divide the hyphae into separated cells [18].

The parameters that lead to germination are not yet well understood. However, *in vitro* studies demonstrated that this behavior is induced by cultivation in serum, complex or chemically defined media; temperatures of 37 °C and pH of around neutrality. On the other hand, yeast growth is stimulated by temperatures at 25 °C, acid pH and glucose as a sole carbon source [27].

Macroscopically, *Candida* spp. colonies on the routinely used Sabouraud dextrose agar (SDA) range in color from cream to yellowish. Depending on the species, their texture may be smooth, pasty, glistening or dry, wrinkled and dull [28]. The use of a differential chromogenic medium such as CHROMagar allows distinguishing between different *Candida* spp. colonies by color, as a result of distinct biochemical reaction. Table 1 summarizes some of the properties that allow the identification of the most relevant *Candida* species.

Table 1. Morphological characteristics of the most relevant *Candida* species. Adapted from [24].

Species	Yeast size (µm)	Hyphae/ Pseudohyphae	Germ tube	CHROM-agar reaction
C. albicans	4-6 x 6-10	+/+	+	Blue-green
C. glabrata	1-4	-/-	-	White, Pink-purple
C. parapsilosis	2.5-4 x 2.5-9	-/+	-	White

As regards the biochemical reactions, it is known that *C. albicans* can ferment and/or assimilate numerous sugars, with the exception of sucrose; *C. glabrata* ferments and assimilates only glucose and trehalose; and *C. parapsilosis* is unable to ferment maltose [21, 29].

1.1.2. Biofilm forming ability

Candida species have an important role in the course of an infection. Their pathogenicity is related with a number of virulence factors, including (i) the ability to express specific host recognition and adhesion biomolecules; (ii) biofilm formation; (iii) secretion of tissue-damaging hydrolytic enzymes (proteases, phospholipases and lipases, and haemolysin) that are responsible for tissue invasion and destruction, as well as for changes on host immunity response [5, 24]; (iv) their versatility and ability to survive in several anatomical sites [27]; and (v) the morphological transitions between yeast and filamentous form (hyphae and pseudohyphae), which plays an important function in tissue invasion and resistance to phagocytosis [30, 31].

The most important virulence factor of *Candida* spp. is the ability to form biofilm on different surfaces, because biofilm cells are not only more resistant to antimicrobial agents than their free-living counterparts, but also appear to be able to resist the host's defense mechanisms [32, 33]. Both in the environment and during the course of infection, organisms frequently exist in the form of biofilms rather than independent entities. Biofilms are the main cause of microbial infections in humans, being involved in approximately 80 % of all infections [34]. Indeed, it was reported that *Candida* strains with a high ability to form biofilms are usually more virulent than others [35].

Biofilms are organized microbial communities characterized by sessile cells embedded in a self-created protective extracellular matrix (ECM) composed by diverse polymeric substances such as cell wall glycoproteins and polysaccharides. This structure promotes biofilm adhesion and formation, keeps its integrity, protects cells from phagocytosis, and limits the diffusion of drugs [36, 37]. Biofilms have a distinct phenotype from those of their planktonic counterparts, which is related to the transcribed genes and growth rate [38, 39]. Within biofilms, cell express genes implicated, for example, in the adhesion to host surfaces (such as agglutinin-like gene) that are not expressed in planktonic cells [40]. Furthermore, microbial cells within biofilm are under the control of signaling molecules and therefore have a collective behavior. This improves not only the access to nutrients and niches, but also their collective defense against other organisms [41].

The primary step in the biofilm formation is adhesion between *Candida* species and host cells/abiotic surfaces (Figure 1). Attachment of *Candida* species cells to materials is mediated by non-specific interactions, such as hydrophobic and electrostatic forces, as well as by specific adhesin-ligand bonds [42–44]. It is important to note that cell hydrophobicity and charge depend on cell growth

morphology and cell surface structure [45]. Attached cells proliferate to form microcolonies and start to deposit the ECM. Then, biofilm goes through a maturation phase until form a thick layer of matrix involving a complex network of yeasts cells, hyphae and pseudohyphae. At a certain time, cells are detached from biofilm, being able to form new biofilms in the surrounding environment.

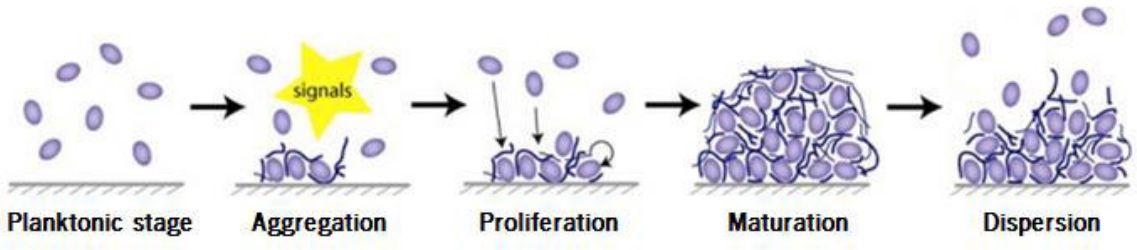

Figure 1. Stages in the formation of a *Candida* spp. biofilm. Adapted from [46].

There are too many reasons for the resistance of *C. albicans* biofilms to antifungal agents: (i) extracellular matrix acts as a diffusion barrier, inhibiting antifungal diffusion to the inner layers of the biofilm; (ii) slow growth rate (due to the limited availability of nutrients, particularly at the base of the biofilm) leads to slow or insufficient drug uptake; (iii) the presence of a small number of "persister" cells that present a high level of drug resistance; (iv) the presence of efflux drug pumps; and (v) alterations in membrane sterol composition [33, 47–49].

It is known that the formation of mature biofilms and consequent production of ECM is strongly dependent on different factors, namely, species, strain, and environmental conditions such as medium composition, oxygen concentration and pH [50, 51]. In fact, a previous study showed that, in the presence of urine on silicone surfaces, *C. glabrata* is able to produce a higher biofilm biomass, in comparison with *C. parapsilosis* and *C. tropicalis* [52]. However, when grown in rich culture media (SDA), biofilm formation by *C. glabrata* was lower compared with other NCAC species [53, 54].

1.1.3. Conventional antifungals

There are several substances that can be applied in the treatment of candidiasis. Each antifungal class utilizes a different way to inhibit or kill fungal pathogens. Azoles interfere with the enzyme lanosterol demethylase (responsible for the conversion of lanosterol to ergosterol), leading to the depletion of ergosterol in the membrane. These antifungal agents, in particular fluconazole, are the

most frequently used in the treatment of candidiasis [55, 56]. Polyenes interfere with ergosterol and consequent with stability/permeability of fungal cell membrane, causing the leak of cytoplasmic content. Echinocandins inhibit the synthesis of ß-glucan, a fundamental component of the fungal cell wall [7], and pyrimidines act on enzymes that regulate the synthesis of nucleic acids [57, 58]. Amongst these antifungals agents, only amphotericin B (a polyene) and echinocandins (such as caspofungin) have showed consistent activity in the *in vitro* treatment of *C. albicans* biofilms [59]. However, even with these agents, infections related with *Candida* biofilms are extremely difficult to eradicate [60].

The efficiency of conventional therapies in the treatment of fungal infections has decreased, as a result of (i) the increase in antifungal drug resistance; (ii) the frequent recurrence of infections; (iii) the time-consuming of conventional treatments; (iv) the organ dysfunction which prevents the use of some agents; and (v) drug-drug interactions, which results from the narrow spectrum of action and toxicity of drugs [7, 61]. The high prevalence of drug resistance has increased the need for development of new effective antifungal therapies.

1.2. Antimicrobial Photodynamic therapy (APDT)

The combined use of visible light and dyes for inactivating microorganisms was first demonstrated more than 100 years ago, when Oscar Raab reported the lethal effect of acridine hydrochloride and visible light on the protozoan *Paramecia caudatum* [62]. Raab and his professors Joldlbauer, Jesionek and Von Tappeiner discovered that acridine was a photosensitizing agent [63]. They created the term "Photodynamic reaction" and eventually, by the early 1900s brought this knowledge to the clinic [64, 65] with brilliant results on cutaneous tumors. In the 1970s, photodynamic therapy (PDT) began to be explored for the selective destruction of cancer [66]. Since then, PDT has emerged as a tool for the treatment of different malignancies (neck, prostate, brain and mesothelioma), non-melanoma skin cancer (basocelullar carcinoma and actinic keratosis), choroidal neovascularization in age-related macular degeneration, lung and Barrett's esophagus cancer, oral lesions, ophthalmologic disorders and autoimmune diseases, such as psoriasis [67–70].

The potential of PDT against diseases of microbial origin was not exploited for several decades due to the discovery of antibiotics in the 1950s and the low efficiency of photodynamic killing of some pathogens, in comparison with the treatment of cancer [7, 71]. However, in the decades of 80 and

90, the interest in the antimicrobial effects of PDT has recovered [72–77] and it has been suggested as a therapy for a variety of localized infections, due to the significant increase in the occurrence of microbial infections.

Antimicrobial PDT (APDT) is an effective method for eliminating microorganisms, in particular those which exist in the form of biofilms, leading cause of microbial infections in humans [7]. This therapy involves the use of harmless visible light combined with a non-toxic and light-sensitive dye — the photosensitizer (PS) — and oxygen. After irradiation with the light of appropriate wavelength, the PS is energized to an excited state that can undergo molecular collisions with oxygen, leading to the generation of reactive oxygen species (ROS) that are responsible for the cell death [62, 78].

There are numerous advantages associated with APDT: (i) broad spectrum of action, with the efficient inactivation of antibiotic-resistant strains; (ii) utilization of low cost light sources [6]; (iii) selectivity to the target cell and absence of mutagenic effect in host tissue [7, 79]; (iv) the mechanisms of photodynamic killing are not target-specific, consequently resistance to this process is improbable to happen, even after multiple treatments [80–82]; (v) capacity of destruction of biofilm's structure, as a result of direct effect of ROS in polysaccharides and glycoproteins present in extracellular matrix [83–85]; (vi) does not lead to the accumulation of toxicity [86, 87]; and (vii) the combination with conventional therapies can lead to significant additive benefits [88]. Nevertheless, APDT can have some side effects, such as long-lasting skin photosensitivity, systemic and metabolic disturbances, and excessive tissue damage at the treated site [89].

1.2.1. Photophysical and photochemical PDT mechanisms

Photosensitizer compounds have a stable electronic configuration, which consists of a singlet state in its ground energy level, whereby all of its electrons are spin paired in low energy orbitals. Upon application of light with wavelength corresponding to the absorption peak of the PS, the electron in the highest occupied molecular orbital (HOMO) of the PS is excited to the lowest unoccupied molecular orbital (LUMO) and keeps its spin. PS reaches the excited singlet state, which is extremely unstable and short-lived (nanoseconds) [34]. The PS can return to the ground energy level through the emission of a photon (fluorescence) or by internal conversion with loss of energy as heat. Alternatively, the excited singlet state PS can undergo intersystem crossing whereby the spin of the excited electron inverts, leading to the formation of excited triplet state [2]. The triplet state is less

energetic than the excited singlet state, but has a considerably longer lifetime, in the range of microsecond to millisecond [2, 90, 91]. In the triplet state, the PS molecule can emit light (phosphorescence) by returning to the ground state or act as a mediator of one or both of two pathways - Type I and Type II reactions - both of which require oxygen (Figure 2) [78].

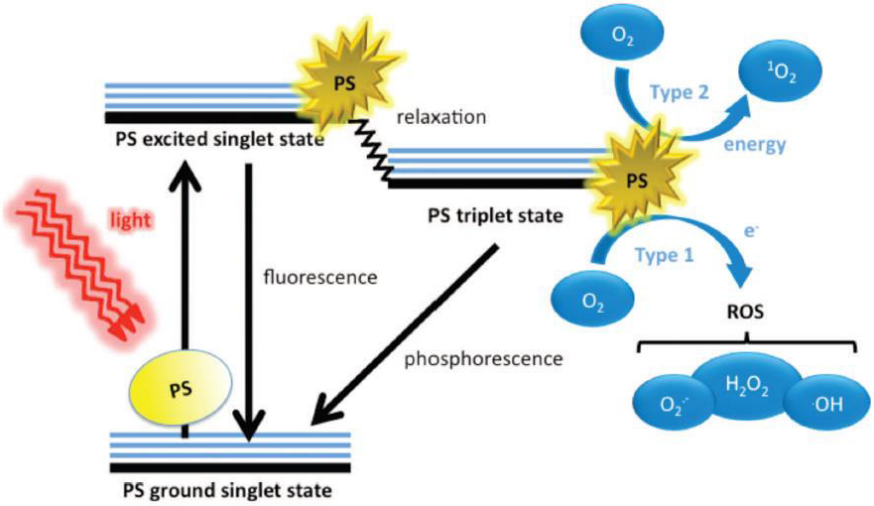

Figure 2. Photophysical and photochemical mechanisms of PDT [34].

Type I

The Type I mechanism involves electron/hydrogen transfer reactions from the PS with the participation of biological macromolecules (including lipids, proteins and nucleic acids) to produce radical ions, which further interact with molecular oxygen to produce ROS, such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) [89, 92]. Type I reactions do not necessarily need oxygen to cause cellular damage, due to the action of free radicals [93].

$O_2^{\cdot-}$ is the ROS more abundant in nature and is formed by transfer of an electron from the PS to oxygen molecule in the ground state. This ROS *per se* may not have sufficient reactivity to kill cells. However, it is considered toxic because it is involved in the formation of other reactive ROS, in particular $^{\cdot}OH$ and peroxynitrite anion (ONOO-, short-lived and highly reactive) by reaction with nitric oxide [94, 95]. $O_2^{\cdot-}$ can also abstract electrons, forming a peroxide ion that instantly abstracts protons to form H_2O_2 [34].

H_2O_2 has a long half-life and capability to diffuse across cell membranes. This ROS can inactivate enzymes, mainly by oxidation of essential thiol groups. However, H_2O_2 is poorly reactive and its toxicity is due in large part to the formation of the $^{\cdot}OH$ in the presence of transition metal ions, such as iron

and copper, and the interaction with $O_2^{\cdot-}$ [96, 97]. The reaction between H_2O_2 and ferrous iron is known as the Fenton reaction (Equation 1).

$$H_2O_2 + Fe^{2+} \longrightarrow OH^- + {\cdot}OH + Fe^{3+} \qquad \text{(Equation 1)}$$

and consists in the homolytic fission of the oxygen-oxygen bond in H_2O_2 to yield a hydroxide ion and ·OH via the oxidation of ferrous iron to ferric iron [98].

·OH is short-lived and shows a small diffusion capacity. However, this is the most reactive of the three ROS formed and, in theory, can oxidize any organic molecule. This ROS acts on molecules near the location where it is formed, essentially lipids, being able to remove hydrogen of double bonds of fatty acids present in cell membranes - lipid peroxidation [99]. In addition, ·OH induces breakage and modification of deoxyribonucleic acid (DNA) bases [100]. ·OH can abstract electrons to become a hydroxide ion, which then can obtain a proton and form water [34].

Type II

In the type II reaction, which is considered the dominant process in PDT [101], the triplet state of PS can directly undergo energy exchange with molecular oxygen (triplet ground state oxygen) to form excited state singlet oxygen, $^1O_2$, which can oxidize a large number of biological substrates, such as proteins, nucleic acids and lipids, and lead to cytotoxicity [102–104]. $^1O_2$, probably the major damaging species in PDT [12, 105], has a short lifetime and high reactivity. It is a non-specific oxidant agent that reacts with double bonds and sulfur moieties and can interact with aromatic components of macromolecules [106, 107].

Because ROS have very limited diffusion distance and a short lifetime, the representative damage action is focused on the targets adjacent to the PS [108]. Therefore, to a certain degree the type of photodamage depends on the precise subcellular localization of the PS within the cells [89]. Figure 3 compares the lifetime, diffusion distance and reactivity of $O_2^{\cdot-}$, H_2O_2, ·OH and $^1O_2$.

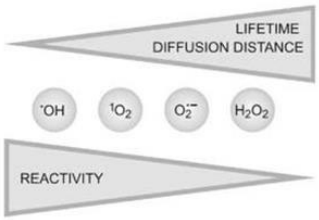

Figure 3. Comparison of lifetime, diffusion distance and reactivity of different ROS [109].

1.2.2. Mechanism of microbial inactivation

Microbial cells are characterized by marked differences as regards the cell size and composition, which has obvious effects in the interaction of exogenously added PS with cell constituents, affecting the efficiency of the photoinactivation process [6]. Thus, the procedure adopted for the treatment of infections must be able to efficiently act on microbial pathogens with very different characteristics. The mechanism of action of APDT starts with a time period of PS action to promote its concentration in/around the target cells, to avoid the damage of host cells or disturb the residual flora of the surrounding tissue. This step is followed by light delivery in a spatially confined and focused manner [7, 89].

The photodynamic mechanism of fungal cells inactivation occurs by perforation of the cell wall and membrane with APDT-induced ROS thereby allowing the translocation of the PS into the cell [110]. Once inside the cell, PSs are distributed to different subcellular targets such as the mitochondria, vacuoles, endoplasmic reticulum, Golgi apparatus and plasma membranes [111]. Oxidizing species generated by light excitation induce photodamage of the different subcellular targets and finally cell death. The risk of DNA damage in eukaryotic fungi is further reduced by the presence of the nuclear membrane that acts as a barrier to the penetration of dyes and their photoproducts [112]. The efficiency of photodamage depends on the type, dose, incubation time and localization of the PS; the oxygenation state of the tissue; the wavelength of light; the light power density (mW cm^{-2}) and the light energy fluence (J cm^{-2}). The abundance of subcellular targets in fungi should reduce the risk of selection of resistant strains.

1.2.3. Photosensitizers

In the 3 past decades, a great deal of work has been carried out in order to understand the correlation between antimicrobial photodynamic efficiency and structure of the photoactive compounds. This resulted in the development of a variety of natural and synthetic PSs [113]. As a rule, PSs are usually organic delocalized aromatic molecules that comprise a large conjugated system of double bonds (that may be considered as a central chromophore) and auxiliary branches (named auxochromes) that are responsible for electron delocalization of the PS, altering its absorption spectra [110, 114]. The choice of the ideal PS should take into account the characteristics of microbial cells, the medium to be treated, and the penetration depth of light into the skin [93].

1.2.3.1. PROPERTIES OF PHOTOSENSITIZERS

The efficiency of photosensitization is critically dependent on physicochemical properties of PSs. The lipophilicity/hydrophilicity balance, the degree of ionization, and the presence of electric charged groups, for example, are extremely related with the mechanisms of PS uptake and the pattern of its distribution within the cells [115].

The hydrophilic character is a very important factor because it avoids self-aggregation of PS. However, hydrophilicity also limits the penetration of PS through the phospholipid bilayer of the plasma membrane, resulting in a decrease of intracellular PS levels. Therefore, the best PSs are usually amphiphilic compounds, with both hydrophilic and hydrophobic regions [113, 116].

It is important to note that the photosensitizing ability of the dyes depends on the target microorganism. Some PSs that are effective against fungi do not have the same effect on bacteria. Cationic PSs are much more efficient in killing Gram-positive and Gram-negative bacteria than their anionic or neutral congeners [117, 118]. As regards fungi, both cationic and anionic types exhibiting efficient phototoxicity [119, 120]. While cationic PSs accumulate in mitochondria, PSs with one or two anionic charges localize in the perinuclear region, vesicles of the cell, and vacuoles [121–124]. However, cationic PSs should be used instead of anionic ones, since the latter exhibit facile uptake by mammalian cells [125]. As a general rule, uptake of exogenous substances by fungi is negatively influenced by lipophilicity and positively affected by hydrophilicity and the presence of electric charged groups [2, 126].

Upon irradiation, all PS are prone to chemical changes or even degradation. This results from a direct attack of singlet oxygen or other ROS on the PS molecules and can be detected by a lowering of absorbance or fluorescence emission [127]. Photodegradation is the chemical destruction of a PS in such a way that the molecule is broken into small fragments, which do not absorb in the visible region of the spectrum. Therefore, photodegraded PSs lose their function in the photodynamic process [128, 129]. On the other hand, photomodification (also referred to as photoproduct formation) consists of small changes in side groups and/or the molecular skeleton of the PS, which generally results in the loss of photodynamic reactivity. The repeated addition of PS during the irradiation period can help overcome this limitation [80]. Nevertheless, there are some cases (particularly that of PpIX) where photoproducts are more effective PSs than their starting molecules [129–131]. Photomodification of PS may also be an advantage, because it can reduce the

generalized photosensitivity after PDT, and increase treatment selectivity (as it decreases the PS concentration in healthy tissues) [113]. Upon light exposure, the intracellular distribution pattern of a PS can change. This phenomenon, which is termed photorelocalization, is important because the location within the cell affects the efficiency of PDT [132, 133].

In APDT, a PS ideally should possess the following properties [6, 113, 134, 135]: (i) pure, stable and soluble in the body's tissue fluids; (ii) absence of toxicity and toxic by-products; (iii) selective accumulation on microbial cells; (iv) a broad spectrum of action in order to act against a wide range of pathogens; (v) a high extinction coefficient is required to increase the number of photons absorbed, which in turn decreases the amount of PS required for a certain effect; (vi) a strong absorption in the red/near infra-red regions of the visible spectrum where light penetration into tissue is maximum; at the same time, this avoids generalized photosensitization by sun light (400-600 nm); (vii) absorption beyond the range of the fungal pigment; (viii) a high photostability to minimize photobleaching; (ix) a high triplet state lifetime; (x) a high quantum yield of triplet state to obtain large concentrations of the activated drug; (xi) a high quantum yield of singlet oxygen; (xii) it should not self - aggregate in the body, because the aggregation decreases the triplet state and singlet oxygen quantum yields; (xiii) tolerable by the body in case of overdose; (xiv) pharmacokinetic elimination from the patient should be rapid, to avoid the necessity of post-treatment protection from light exposure and prolonged skin photosensitivity; and (xv) it should be easily produced in large-scale to make it cost-effective and widely applicable.

1.2.3.2. THE MOST COMMONLY USED ANTIMICROBIAL PHOTOSENSITIZERS

A large number of PSs have been used over the years as antimicrobial agents in APDT. Because some degree of tissue penetration is required to destroy pathogens localized below the skin surface, the development of compounds with a strong absorption in the red region of the visible spectrum, where light penetrates 3.0 mm down the tissue (light in the blue region penetrates only 1.5 mm), has been the main objective of the studies in this field [2].

Nowadays, the major PS classes employed on APDT field include phenothiazines, cationic tetrapyrroles such as porphyrins and phthalocyanines, as well as 5-aminolevulinic acid (ALA), which, while not a PS in itself, acts as a porphyrin precursor (Figure 4) [110]. The groups reveal different photochemical and photophysical properties and therefore have different mechanisms of action.

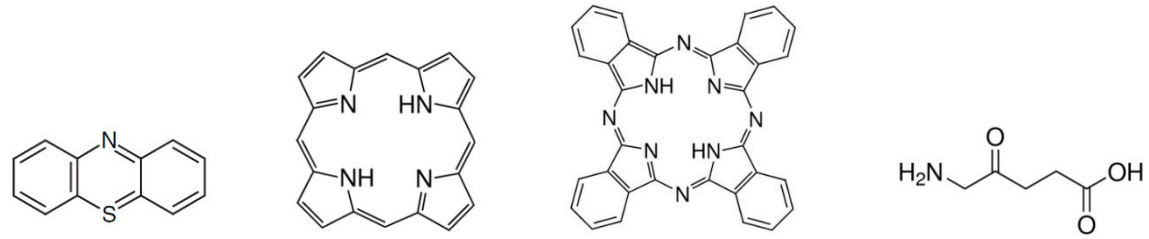

Figure 4. Basic chemical structures of the main groups of antimicrobial PSs. Adapted from [2, 6, 136].

Phenothiazines

Phenothiazines have a single cationic charge that is delocalized over the three-ring planar structure and its maximum absorption wavelength is in the range of 600-900 nm [137]. Within these group, methylene blue (MB) and ortho-toluidine blue (TBO) are the most widely used compounds [138–140] and the maximum absorption wavelength in water is 656 nm for MB and 625 nm for TBO [141]. Both compounds show a high efficiency in singlet oxygen production [142]. New MB (NMB) and dimethyl-MB are also often used and are even more powerful antimicrobial agents than MB and TBO [143]. Phenothiazines are usually localized in plasma membrane of yeasts and lead to cell death by increasing its permeability [144].

Porphyrins

Porphyrins are derivatives of porphin, the name given to the tetrapyrrole ring structure, and are widely encountered in nature. These PSs contain four pyrrole subunits connected by four methane bridges [136, 145, 146]. Their structure varies depending on the number and type of side groups, particularly carboxylic acid groups, being classified into: uroporphyrin (eight carboxylic acid groups); coproporphyrin (four) and protoporphyrin (two), and the optimal wavelength to photokilling is approximately 410 nm [147].

Several new synthetic porphyrins derived compounds have been produced in order to get PSs with a maximum absorption wavelength of 650-700 nm, and high singlet oxygen quantum yields [110]. These compounds are usually associated with polar hydrophilic groups (such as carboxy, sulfonic, hydroxy, quaternary ammonium, or pyridinium), which facilitate the PS uptake and, consequently, the efficiency of photodynamic process [148].

Phthalocyanines

Phthalocyanines have a structure in the form of condensates benzene rings and are characterized by high singlet oxygen quantum yields and high extinction coefficient in the far red (680 - 720 nm) spectral region [115, 149]. The singlet oxygen production is increased by the presence of a central atom (zinc, silicon or aluminum), which plays an essential role in the photobiological activity, influencing the triplet state lifetime and quantum yield; as well as the introduction of polar substituents on the side chains, which affects the polarity of PS. As they are essentially hydrophobic compounds, these PS are usually encapsulated in liposomes in order to improve their uptake by cells. Within the cells, these PSs are usually accumulated in mitochondria and apparently induces apoptosis, substantiated by increased externalization of phosphatidylserine and DNA fragmentation [110, 150, 151].

ALA

ALA is not a PS by itself but all eukaryotic cells metabolize it into a very active endogenous PS, protoporphyrin IX (PpIX), which is then converted to heme via biosynthetic enzymes present in mitochondria and cytoplasm [152]. When a large amount of exogenous ALA is applied, PpIX accumulates inside the cells because the final step in heme formation by enzyme ferrochelatase is a rate-limiting step [153]. This factor has been exploited by APDT. Upon irradiation, PpIX induces cytotoxic effects that damage essentially the mitochondria, where PpIX is synthesized, and plasma membranes. Prolonged treatment can have widespread effects on cytoplasmic structures and interfere with DNA and ribonucleic acid (RNA) synthesis [154].

1.2.3.3. BENZO[A]PHENOXAZINES

Phenoxazines are heterocyclic compounds consisting of two benzene rings fused to oxazine. They are analogous in structure to phenothiazines with an oxygen atom in place of sulfur atom (Figure 5). The addition of benzene rings to phenoxazines leads to the formation of benzophenoxazines that can be angular (benzo[a]phenoxazine and benzo[c]phenoxazine) or linear (benzo[b]phenoxazine) depending on the orientation of benzene ring fusion [155].

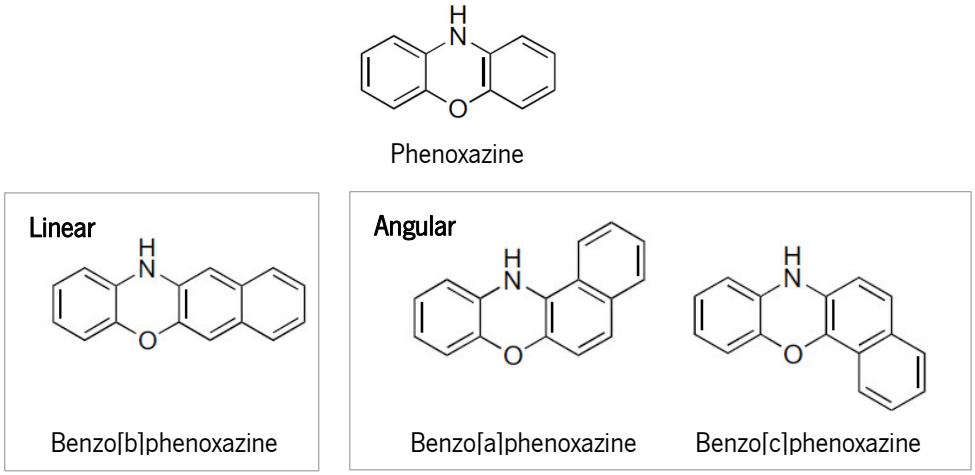

Figure 5. Chemical structure of phenoxazines and benzophenoxazines. Adapted from [155].

Benzo[a]phenoxazines have been widely used as fluorescent probes in different applications, such as for checking hydrophobic surfaces in proteins, as lipid stains in membranes and also to study the interaction between the probe and DNA and its application in electrochemical recognition [156–158]. However, benzo[a]phenoxazines may have other applications. Nile blue, which belongs to this family, was employed as a lead compound in PS development for cancer PDT by Foley *et al.* [159], mainly due to its biological selectivity for tumoral tissues and quickly pharmacokinetic elimination [160]. As a result of investigation on this field, there have been created some Nile blue derivatives that possess different physicochemical and photochemical properties, in order to enhance the sensitizer triplet state and singlet oxygen quantum yields [161]. Benzo[a]phenoxazines also possess many of the chemical and physical properties that characterize an effective antimicrobial PS. They are hydrophilic, cationic dyes; stable at physiological conditions; binds strongly to both bacteria and fungi; effective in targeting intracellular organelles; efficiently absorb red light; and possess a broad-spectrum of action, a high singlet oxygen quantum yield, and a high phototoxicity [137, 162, 163]. The development of improved PS derived from Nile blue is a promising approach to create new PS for use as antimicrobial drugs in the treatment of localized infections.

1.2.3.4. NEW FRONTIERS IN APDT

A lot of compounds have been often proposed as antimicrobial PSs: chlorins, bacteriochlorins [89, 164], halogenated xanthenes (Rose Bengal) [165], perylenequinones (hypericin) [166], cationic buckminsterfullerenes (C_{60}) [167, 168] and psoralens (furanocoumarins) [169].

PSs based on the tetrapyrrole nucleus, such as porphyrins, chlorins, bacteriochlorins and phthalocyanines are lipophilic compounds and easily form aggregates in aqueous solution, leading to the loss of photodynamic efficiency [170, 171]. In the last decades, different procedures of PS delivery have been explored in order to overcome this problem, including the conjugation of PSs with cationic polymers [172] or monoclonal antibodies [173], which improve PS kinetics, and its encapsulation in cationic nanoparticles [174, 175] or liposomes [170, 176]. Among these biologic conjugates, liposomes are the most used and have been proved to enhance the killing effect of numerous PSs. They can increase the stability and solubility of PS, and facilitate its penetration into microbial cells by fusion processes or disturbing the cell walls [177]. The efficiency of photosensitization can also be enhanced by covalent binding of PS with bacteriophages [178].

1.2.4. Light sources and delivery

By definition, PDT needs a source of light to supply the requisite energy for singlet oxygen production, or to promote redox reactions. Ideally light sources should exhibit suitable spectral characteristics that coincide with the maximum absorption wavelength of the PS [133].

The spectrum of the light used for excitation of PS is another critical issue. Visible light, which covers the range of 400-700 nm, is most relevant to PDT. When used *in vivo*, emission in the ultraviolet and infrared region should be avoided due to the risks of mutagenesis and heating of tissue, respectively [2]. In practice, the range of light used is generally longer than 620 nm, because light in the far red region of the spectrum penetrates more easily than lights of shorter wavelength [179]. On the other hand, at wavelengths shorter than 600 nm endogenous molecules have a strong absorption and therefore capture most of the incoming photons [136]. Nevertheless, PSs with absorption in the blue region can be useful, for example, for the treatment of *Candida* species that invade only the stratum corneum [126].

1.2.4.1. THE CONCEPT OF IRRADIANCE AND FLUENCE

Irradiance, also termed light power density, is the radiation power (Watt) per unit area (usually cm^2) that is received at a surface. Energy fluence, or simply fluence, is the energy (Joule) per unit area (usually cm^2) delivered in a given time interval. Power outputs of light sources used in antifungal PDT are typically in the range of 10–100 mW cm^{-2}. To avoid excess of heat, the total light dose should be

less than 200 J cm^{-2} [7]. In fact, the high power of light is of minor importance in PDT, because relatively low intensities are often sufficient to induce the killing of *C. albicans* and bacteria cells [180, 181].

The success of PDT depends upon the irradiation of PS with light of sufficient intensity. Thus, it is convenient to predict the behavior of light within the target tissue. Light fluence decreases exponentially with thickness of the tissue, and this decrease is determined by two main factors: light absorption by endogenous tissue chromophores (hemoglobin, myoglobin and cytochromes) and light scattering (which is enhanced by the irregular shape of the tissue and the presence of hair follicles and glands) [182]. The extent of both processes depends on the optical properties of the tissue and wavelength of incident light [86].

1.2.4.2. THE MOST COMMONLY USED LIGHT SOURCES

Lasers (Light Amplification by Stimulated Emission of Radiation) are the most preferred sources of light used in PDT. Nevertheless, filtered incoherent lamps have also been employed. The choice of the most appropriate light source can be dictated by the location, size and morphology of the target tissue, as well as by the choice of PS and the associated cost [179].

Lasers

Lasers produce high energy monochromatic light of a specific wavelength with a narrow bandwidth for a specific PS. The laser light can be focused passed down an optical fiber and directly delivered to the target site. The most widely used lasers in PDT applications are shown in Table 2.

Table 2. Lasers most commonly used in PDT. Adapted from [179].

Light source	Wavelength (nm)	Irradiance (mW cm^{-2})	Bandwidth (nm)
Argon laser	488 and 514,5	500 - 1000	Monochrom (5-10 nm)
Metal vapor laser	UV or visible (depending on metal)	Up to 10000	
Solid state	1064; 532; 355; 266	Up to 10000	
Semiconductor diode laser	600 - 950	Up to 700	Monochrom

The advantages of the use of lasers are related with (i) the maximum effectiveness provided by monochromatic light, if the wavelength of the laser corresponds with the maximum peak absorption of the PS; (ii) the therapeutic exposure time is minimized by high irradiance; (iii) delivery of light to otherwise inaccessible locations; and (iv) its focal precision prevents damage of the surrounding tissue. The use of lasers has drawbacks, because they are expensive, require special maintenance and can only be used in small areas of the skin surface [179].

Lamps

Non-coherent light sources can also be used in PDT. These polychromatic lights can be directly delivered to the target cells without the need to be coupled to an optical fiber. Due to the large illumination fields, these light sources could be used in the treatment of large skin lesions, which is an advantage over lasers.

Furthermore, when compared to lasers, lamps are easier to use and less expensive. The broad spectrum of radiation emitted by lamps allows the use of a wide range of PSs with different absorption maxima within the emission spectrum of the lamp. These light sources can also be used in conjunction with optical filters to transmit light of just one wavelength. The disadvantages of conventional lamps include significant thermal effect, low light intensity and difficulty in controlling light dose [179]. The most widely used lamps in PDT are shown in Table 3. The bandwidth of the different light sources is dependent on the filters used.

Table 3. Lamps most commonly used in PDT. Adapted from [179].

Light source	Wavelength (nm)	Irradiance (mW cm^{-2})	Bandwidth (nm)
Tungsten filament	400 - 1100	Up to 250	10 - 100 (depending on filters used)
Xenon arc	300 - 1200	Up to 300	
Metal halide	250 – 730 (depending on the metal)	Up to 250	
Sodium (phosphor coated)	590-670	Up to 100	10-80 (depending on filters used)
Fluorescent	400 - 450	Up to 10	≈ 30
Light-emitting Diode (LED)	400-1000	Up to 150	5 - 10

1.2.5. Clinical applications

APDT has been widely employed in the treatment of several diseases. For clinical applications, an ideal antimicrobial photodynamic inactivation should exhibit extensive killing of the pathogens with minimal damage to host tissues. Indeed, some studies have demonstrated that combination of an appropriated incubation time with low PS levels or slight irradiation parameters can effectively kill microbial cells without cause *in vitro* toxicity or DNA damage to keratinocytes or fibroblasts [112, 183–188].

A study of Dovigo *et al.* [189] demonstrates that curcumin-mediated APDT can be phototoxic to macrophages (leading to a significant decrease in cell metabolism of around 86 %) when using the same parameters as used for fungal cells. However, this therapy was even more effective in the inactivation of yeast cells than the defense cells. Thus, APDT continues to be an interesting approach in the treatment of superficial and cutaneous diseases, particularly those of the skin and oral cavity. Nevertheless, it is important to carry out further investigations in order to increase the selectivity to microbial cells and prevent the damage of mammalian cells.

At present, APDT appears to be especially convenient for the treatment of localized infections, such as periodontitis, oral candidosis or chronic wounds. Most of the clinical applications involve topical administration of ALA or the ALA-methyl ester (MAL) [190–195]. The combination of MB or Toluidine Blue (TB) together with red light is also used in different clinical applications, such as the disinfection of blood products, sterilization of dental cavities and root canals, and treatment of periodontitis [84, 196]. The development of new classes of PSs, the improvement of dosimetry and further enhancement in technology may drastically change the currently achieved APDT clinical outcome. Clinical trials are desirable to evaluate APDT efficacy, mainly in comparison with existing treatments. However, there is a lack of consensus in relation to skin preparation, incubation time, type of light source, and time of exposure to light. For this reason, is imperative the development of standardized treatment protocols, through the application of APDT in the several conditions [70].

1.2.6. *Candida* species inactivation by APDT

APDT has been effectively applied against *Candida* species in planktonic or biofilm form and, in most cases, complete kill of sensitive or resistant pathogens have been readily achieved. The higher

resistance of biofilms of *Candida* compared to planktonic cells was verified by several authors [189, 197, 198].

There is an expanding body of literature regarding APDT-based *Candida* inactivation strategies, with emphasis in the use of different PS. Several classes of PS, both natural and synthetic, have been tested for antifungal PDT, most of them belonging to the phenotiazinium and porphyrin classes. TBO has been successfully used as a photosensitizing agent to inactivate *C. albicans* [120], being less cytotoxic than other photosensitizing agents. However, *in vitro* studies showed that NMB (characterized by more lipophilic nature than traditional MB) has a higher activity against *C. albicans,* when compared with TBO and MB [199].

Photofrin (Porfimer sodium), Photogem, Rose Bengal, Al(III)-tetrasulphonated phthalocyanine, malachite green and erythrosine can also be used to inactive *C. albicans* [115, 200–204]. However, some of these PSs, in particular malachite green, seem to be less effective than MB and TBO in reducing *C. albicans* viability [203]. Low concentrations of curcumin (a natural pigment isolated from rhizomes of *Curcuma longa*), and low incubation times and dose light also shown to be effective in eliminating *Candida* species, both in planktonic and biofilm forms [205].

APDT is a developing area of research and most of the studies performed in this field are *in vitro* experiments. Nevertheless, there are some *in vivo* studies. Junqueira *et al*. [206] observed that rats treated with MB and laser light developed less candidiasis lesions, compared with the control group. Teichert *et al*. [11] also showed that the application of MB and illumination by diode laser was able to totally eradicate *C. albicans* from the oral cavity of a immunodeficient murine model.

1.2.7. Mechanisms of resistance to APDT

Given that APDT products have no a specific target and can cause damage to a variety of cellular organelles, emergence of resistant microbial cells is unlikely to occur. Nevertheless, many yeasts, including *Candida* species, can use different mechanisms of resistance to antimicrobial therapy. They can, for example, reduce PS uptake or alter its structure; increase the efflux of PS by efflux drug pumps or induce the production of enzymes (catalase and superoxide dismutase) to neutralize ROS [207, 208].

By modifying the treatment conditions, photoinactivation of resistant species can be enhanced. According to Garcez *et al*. [209], the inclusion of an oxidant agent (H_2O_2) in the formulation of MB leads to a higher uptake of this PS and, consequently, increases the potential of inactivation of *C. albicans.*

On the other hand, as some dyes are substrates of drug-efflux systems, the use of specific efflux pump inhibitors added to PS can increase the efficacy of APDT. The two main fungal efflux systems are: adenosine triphosphate (ATP)-binding cassette (ABC) transporters and major facilitator superfamily (MFS). Prates *et al*. [210] observed that both systems affect the efficiency of MB-mediated APDT in *C. albicans*, suggesting that both MFS and ABC are involved in MB export. The authors also reported that the effect of ABC overexpression was more prominent when compared with MFS overexpression in protecting cells against APDT, and that photoinactivation by MB could be enhanced by the ABC inhibitor verapamil and not by the MFS inhibitor INF271. This can be explained by the hypothesis that the MFS channel can also serve as an uptake mechanism of PS. However, it is known that the PS can induce phototoxicity reactions without being taken up by cells, because cell wall and membranes are important targets for APDT.

Biofilms also increase the resistance to APDT and several authors have reported techniques to improve the drug penetration into biofilms and subsequently enhance APDT efficacy [37]. Coleman *et al*. [211] characterized the antifungal activity of saponins, a family of natural compounds composed of sugar moieties binding to a hydrophobic aglycone backbone. When used in combination with PSs, saponins may form pores in lipid bilayers, thereby increasing cell permeability and PS penetration. Saponins are also known to prevent *C. albicans* biofilm formation.

The increase in heat shock proteins (HSP) expression after photodynamic process can also be a mechanism whereby microbial cells acquire resistance to APDT, since HSPs play an important role in refolding proteins and prevention of protein aggregation [212, 213]. St Denis *et al*. [214] reported that upregulation of HSPs DnaK and GroEL after TBO-mediated APDT of *Escherichia coli* and *Enterococcus faecalis* allowed an increase in survival of 2 log for *E. coli* and 4 log for *E. faecalis*. Furthermore, the authors demonstrated that the use of a DnaK inhibitor did not significantly potentiate the killing effect of APDT in both microorganisms.

1.2.8. Oxidative stress

Basal levels of ROS generation in cells are beneficial for a number of physiological functions. In fact, ROS are known to regulate signaling pathways via interaction with the signaling molecules, affecting cell proliferation, differentiation, survival, metabolism and anti-inflammatory responses [215]. ROS are usually produced during normal aerobic metabolism, but can be increased by PDT or addition of chemical compounds like H_2O_2 or paraquat (PQ; 1,1′-dimethyl-4,4′-bipyridylium dichloride). PQ is a quaternary nitrogen herbicide and its toxicity is mediated by $O_2^{\cdot-}$, a reactive specie generated by the reoxidation of reduced PQ by molecular oxygen [216].

To maintain redox-homeostasis, cells have developed elaborate defense mechanisms that neutralize ROS, including ROS-scavenging enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase, peroxidases, and non-enzymatic mechanisms such as glutathione [217, 218]. One of the principal antioxidant enzymes is SOD that is responsible for the conversion of $O_2^{\cdot-}$ into H_2O_2, which can in turn be converted to water and oxygen by CAT [34]. $^1O_2$ and $^{\cdot}OH$ cannot be broken down by enzymatic reactions, but can be quenched by antioxidants, including antioxidant peptides (glutathione) or antioxidant sugars (ascorbic acid) [34, 107]. $^1O_2$ may even inactivate some antioxidant enzymes, for example, CAT and SOD [219].

However, when ROS concentration exceeds the cellular antioxidant defense system, either by increased ROS generation or by decreased cellular antioxidant capacity, oxidative stress occurs and the vital functions of the cell can be severely compromised [215]. ROS induce photodamage of biomolecules and cell organelles by oxidation of proteins, lipids and nucleic acids [220]. The peroxidation of unsaturated fatty acids and cholesterol is initiated by free radicals and results in alteration of the fluidity, permeability and integrity of cell membrane.

Proteins are major targets for oxidative reactions. They can be oxidized either directly by reaction with ROS or by a reaction involving the products of lipid peroxidation. Protein oxidation can occur in the polypeptide backbone, which can result in fragmentation of the polypeptide, or in the amino acid side chains, resulting in the inactivation of the protein. Changes that occur in these molecules can also include di- or multimerization; unfolding and structural alterations, resulting in functional inactivation; and changes in binding of cofactors and metal ions [221]. For some authors the damage of cytoplasmic proteins and mitochondria appears to be the principle cause of cell death [222], unlike the DNA damage.

Damage to DNA has been shown in many *in vitro* studies with PDT. ROS can induce DNA base oxidation, cross-linking of DNA strands or sister chromatid exchange [223–225]. However, the DNA damage is related not only with the parameters of PDT, but also with cellular mechanisms of repair and survival [226]. In addition to intracellular damage of biomolecules, PDT also leads to changes in the extracellular matrix, namely in proteins responsible for cell adhesion, influencing the interaction between cells and cell-extracellular matrix. All these damages result in the cell death by apoptosis or necrosis.

2. CHAPTER II

MATERIALS AND METHODS

2.1. Organisms and culture media

Three different species of *Candida* - *C. albicans* ATCC 90028, *C. glabrata* ATCC 2001 e *C. parapsilosis* ATCC 22019 - were used in this study. All of these species are reference strains from American Type Culture Collection (ATCC). Cells were maintained in Sabouraud Dextrose Broth medium (SDB; Liofilchem®, Roseto degli Abruzzi, Italy) supplemented with 10 % of glycerol and stored at -80 °C.

SDB medium was prepared by the addition of 30 g L^{-1} of SDB in distilled water, followed by sterilization in an autoclave at 121 °C for 15 min. SDA plates were prepared by supplementation of SDB with 20 g L^{-1} of agar (Liofilchem®). Both media were stored at room temperature.

2.2. Antimicrobial photodynamic therapy

2.2.1. Photosensitizers

Two PSs belonging to the class of benzophenoxazines – N-[5-(3-hydroxypropylamino)-10-methyl-9H-benzo[a]phenoxazin-9-ylidene]ethanaminium chloride (FSc) and *N*-(5-amino-9*H*-benzo[*a*]phenoxazin-9-ylidene)-*N*-ethylethanaminium chloride (Nile blue chloride) – and a porphyrin dye – 3,7,12,17-Tetramethyl-8,13-divinyl-2,18-porphinedipropionic acid (PpIX) – were used for the sensitization of *Candida* biofilms. The first one was synthetized in the Centre of Chemistry of University of Minho and kindly provided by Professor Maria Sameiro Torres Gonçalves. Nile blue chloride and PpIX were purchased from Sigma-Aldrich. Chemical structures of the three PSs are shown in Figure 6.

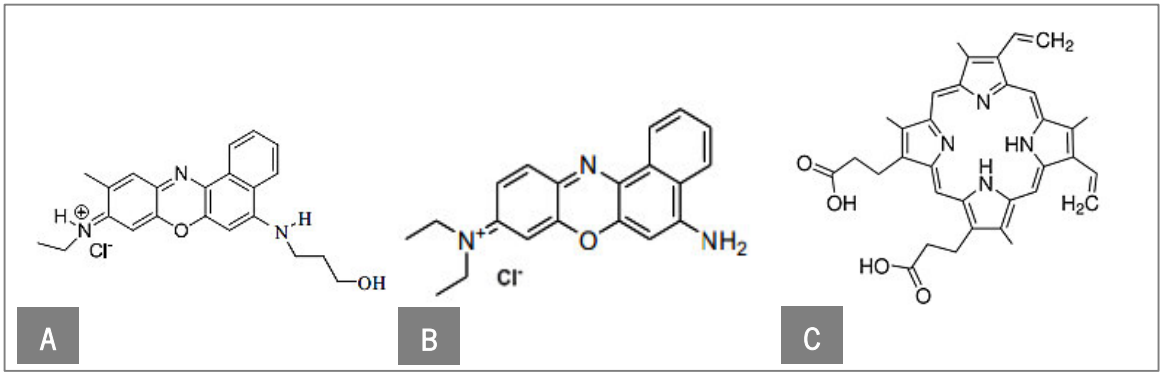

Figure 6. Chemical structure of FSc **(A)**, Nile blue chloride **(B)** and PpIX **(C)** dyes.

Stock solutions of these dyes were prepared by dissolving the powder in 1 mL of Dimethyl Sulfoxide (DMSO), in 1.5 mL sterile brown microtubes (to protect the PSs from the light), at a final concentration of 10 mM. These stock solutions were sonicated for few minutes to promote a great solubilization of the compounds and stored at -17 °C. Before each experience, stock solutions were diluted in phosphate buffered saline solution (PBS; pH 7.5; 0.1 M) in order to obtain several concentrations.

PBS solution was prepared by the addition of 8 g L^{-1} of sodium chloride (NaCl), 0.2 g L^{-1} of potassium chloride (KCl), 0.2 g L^{-1} of dipotassium hydrogenphosphate (K_2HPO_4) and 1.15 g L^{-1} of disodium phosphate (Na_2HPO_4) in distilled water. pH was adjusted to 7.5 and the solution was autoclaved at 121 ˚C for 15 min and kept at room temperature.

The wavelengths of maximum absorption of FSc and Nile blue are 631 nm and 623 nm, respectively [227, 228]. On the absorption curve of PpIX, the highest peak belongs to the Soret band at 375–405 nm. However, the most used wavelength is about 625–633 nm red light, because this light penetrates easily the skin than green or blue light with lower wavelengths [110, 113].

2.2.2. Biofilms formation

For *in vitro* biofilm formation, few colonies of each *Candida* species (previously cultured on SDA at 37 °C for 48 h) were grown in 30 mL of SDB for 18 h, at 37 °C and 120 rpm. Cells were harvested by centrifugation at 3000 x *g*, for 10 min at 4° C and washed twice with 15 mL of PBS. Pellets were re-suspended in 10 mL of SDB and the number of cells in suspension was quantified in a Neubauer haemocytometer (Marienfild, Land-Könicshofen, Germany). Yeasts suspensions were diluted in fresh SDB medium to adjust the cellular concentration to $1x10^7$ cells mL^{-1} and 200 µL of the standard cell suspension was transferred into selected wells of a 96-well polystyrene microtiter plate (Orange Scientific, Braine-l'Alleud, Belgium). The plate was incubated aerobically for 24 h at 37 °C and 120 rpm. At this time, 100 µL of SDB medium was removed and the same quantity of fresh SDB was added to each well. The plate was incubated for more 24 h, under the same conditions. Once completed the 48 h of biofilm growth, the medium was removed and the biofilm was washed with 200 µL of PBS to remove remaining non-adherent cells.

2.2.3. Dark toxicity

Prior to irradiation, biofilms of *Candida* spp. were incubated with different concentrations of PSs in order to evaluate the effect of each dye on cells without the interference of direct light. After biofilms formation, 200 µL of dye solution was added to each well and the plate was incubated in the dark for 18 h at 37 °C and 120 rpm. Negative controls (for cells and dyes) were also carried out, by replacing the PSs for PBS. After the incubation, dye solutions were removed and the biofilms were washed twice with 200 µL of PBS to remove excess of PS. Finally, 200 µL of PBS were added to each well to disrupt the biofilm and quantification of colony forming units (CFUs), as described in the section 2.2.5.

2.2.4. Photodynamic therapy against *Candida* biofilms

After *Candida* biofilms formation and dark incubation for 18 h with each PS, as described above, the dye solutions were removed and the biofilms were washed twice with 200 µL of PBS to remove excess of PS. For the irradiation assay, 200 µL of PBS were added to each well. The effects of both PS and irradiation *per se*, and the control group (treated with PBS in the absence of light) were evaluated.

Candida biofilms were irradiated with a xenon arc lamp (OSRAM HBO 200W) at room temperature. This light source produces a largely continuous and uniform spectrum across the entire visible spectral region and displays a complex line spectrum in the 750 to 1000 nanometer region of the near-infrared spectrum. To carry out this experimental work, a bandpass filter centered on 600 ± 2 nm (ThorLabs, New Jersey, USA) was joined to the irradiation system.

Biofilms irradiation was made top-to-bottom and performed under aseptic conditions. During irradiation, microplates were covered with a black cap, which had an orifice with the same size of wells, in order to prevent the spread of light to foreign wells. Light power was measured with an integrating sphere photodiode power sensor (model S144C; ThorLabs) using the PM100D Utility software (Thorlabs) at the beginning of each experiment in the same location of biofilms irradiation. Type and concentration of PS, as well as irradiation conditions (light power, irradiance, irradiation time and fluence), used in the assays are summarized in Table 4. The area of the biofilm in the bottom of the well is approximately 0.33 cm^2, however the irradiated area was 0.13 cm^2.

Table 4. Parameters used in the APDT assays against *Candida* biofilms.

Candida spp.	PS	Light power (mW)	Irradiance (mW cm^{-2})	Irradiation time (min)	Fluence (J cm^{-2})
C. parapsilosis	50 μM FSc	0.9	7	20	8
	300 μM FSc	0.9	7	20	8
				60	25
	500 μM FSc	1.8	14	60	50
C. glabrata	75 μM FSc	0.9	7	20	8
				60	25
		1.8	14	60	50
C. albicans	300 μM FSc	0.9	7	60	25
		1.3	5 [1]	60	18
			10	60	36
	300 μM Nile blue	1.3	10	60	36
	300 μM PpIX	1.3	10	60	36

[1] The irradiated area in this assay was 0.24 cm^{2}.

Irradiation was followed by the disruption of biofilm and quantification of its viability by the CFU's method, as explained in the section 2.2.5.

2.2.5. Determination of biofilm viability: quantification of CFUs

At the end of the experiments, yeast biofilms were detached from the bottom and the wall of each well by scraping these surfaces with a sterile pipette tip. The content of each well (200 μL) was transferred to a sterile microtube and the procedure was repeated with 100 μL of PBS in order to improve the detachment of biofilm. In the case of dark toxicity assays, the cells that were detached from 3 independent wells per condition were harvested for the same microtube. Each microtube was vigorously vortexed during 30 s to disrupt the matrix of biofilm and serial decimal dilutions were prepared in PBS and 10 μL were plated on SDA in triplicate. After 24 h of incubation at 37 °C, the number of colony forming units per unit area (CFU cm^{-2}) was determined.

2.3. Oxidative stress response

2.3.1. Operating Conditions

Two ROS-inducing agents, H_2O_2 (Fluka) and PQ (Sigma-Aldrich), were used to evaluate the antioxidant response of *Candida* species. Yeast cells from SDA plates (grown during 48 h at 37 °C) were pre-cultured during 18 h at 37 °C and 120 rpm in Erlenmeyer flasks with 200 mL of SDB medium. Cellular concentration was quantified by cell counting and cells were harvested by centrifugation (3000 x *g*, for 10 min at 4° C) and re-suspended in 50 mL of PBS buffer in order to achieve a final cellular concentration of $1x10^7$ cells mL^{-1} (first assay) or $1x10^8$ cells mL^{-1} (second assay). PQ and H_2O_2 were added to cellular suspensions at final concentrations of 2 mM (first assay) and 20 mM (second assay). A negative control (cell suspension without chemical oxidants) was also performed, in the same conditions described above.

2.3.2. Analytical Methods

Yeasts samples were collected after 3 h of exposure to oxidants (at 37 °C and 120 rpm) for analysis of cell viability and antioxidant enzymes activity (SOD and CAT). Cell viability was estimated by the CFU's method. After serial decimal dilutions with sterile PBS, 10 µL of cellular suspension (diluted from 10^{-2} to 10^{-6}) were plated on SDA medium in quadruplicate. After 24 h of incubation at 37 °C, the number of colony forming units per milliliter (CFU mL^{-1}) was quantified.

Total protein and antioxidant enzymes were measured after cell disruption and dialysis of cell extracts. For preparation of cell extracts, cells were harvested from the cultures by centrifugation (3000 x *g*, for 10 min at 4° C) and re-suspended (to a final concentration of $1x10^8$ cells mL^{-1} and $1x10^9$ cells mL^{-1} in first and second assays, respectively) in 700 µL of 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM ethylenediamine tetraacetic acid (EDTA), frozen, and stored at -20°C. Cells were disrupted by mechanical disruption with 0.5-mm glass beads during 10 min (1 min bursts with 1 min cooling intervals). Whole cells and debris were removed by centrifugation at 4000 rpm for 15 min at 4°C. The clear supernatant was dialyzed overnight (14 000 Da cut-off membrane) against 50 mM sodium phosphate buffer (pH 7.8), 0.1 mM EDTA at 4 °C with agitation.

Total soluble protein of cell extracts was determined by Bradford's method [229]. It was measured using the Coomassie Plus – Bradford Assay Kit (Pierce, 23236). The Coomassie Plus Kit is a quick and ready-to-use coomassie-binding, colorimetric method for total protein quantification and is a modification of the well-known Bradford method. When coomassie dye binds to proteins in an acidic medium, an immediate shift in absorption maximum occurs from 465 nm to 595 nm with a concomitant color change from brown to blue. To determine the amount of total protein in the cellular extracts, 300 µL of Coomassie dye were added to 10 µL of sample. The microplate was shaken for a few seconds and incubated for 10 min at room temperature in the dark. The absorbance was measured at 595 nm in an ELISA spectrophotometer (Rainbow, Tecan). Protein concentrations were estimated using the respective calibration curve with bovine serum albumin (BSA) as standard.

SOD activity was measured spectrophotometrically by monitoring the inhibition of pyrogallol (Sigma-Aldrich) autoxidation by SOD, as described by Marklund and Marklund [230]. The reaction mixture (final volume of 1 mL) contained 20 µL of sample, 890 µL of 50 mM Tris-HCl buffer (pH 8.2), 1 mM EDTA and 90 µL of 20 mM pyrogallol (dissolved in 10 mM HCl and prepared immediately before the experiment, in a dark bottle). The increment of absorbance (Abs) was measured in a JASCO V560 spectrophotometer at 420 nm for 60 s with intervals of 5 s. Tris-HCl buffer was used as the blank, and a similar protocol was conducted. Equation 2 was used to calculate SOD activity.

$$SOD\ (U\ mL^{-1}) = \left(\frac{\Delta_{Ab}}{\Delta_A} - 1\right) \times \frac{1000}{V_a} \qquad \text{(Equation 2)}$$

Where Δ_{Ab} is the slope of Abs/s obtained for the blank; Δ_A is the slope of Abs/s for the sample; and V_a is the sample volume used in the reaction. One unit of SOD activity was defined as the amount of enzyme that inhibits by 50 % the velocity of pyrogallol autoxidation at pH 8.2 and 25 °C. SOD specific activity was expressed as units of enzyme activity per milligram of total soluble protein (U mg^{-1}).

CAT activity was determined spectrophotometrically at 240 nm by measuring the velocity of substrate consumption, according to Beers and Sizer [231] method. The decomposition of H_2O_2 is monitored by the decrease of absorbance through the time. The reaction was started by the addition of 25 µL of sample to 667 µL of 50 mM sodium phosphate buffer (pH 7.0) and 333 µL of 30 mM H_2O_2. The assay was performed on quartz cuvettes of 1 mL and the absorbance was measured in a JASCO V560 spectrophotometer for 60 s with intervals of 5 s. Sodium phosphate buffer was used as the blank, and a similar protocol was conducted. CAT activity was determined using the Equation 3.

$$CAT\ (U\ mL^{-1}) = 2{,}31 \times\ m \times (^{V_t}/_{V_a}) \qquad \text{(Equation 3)}$$

Where V_t is the total volume of mixture; V_a is the sample volume; and m is the absolute value of slope of Log (Abs)/s. One unit of CAT activity was defined as the amount of enzyme that catalyzes the decomposition of 1 µmol of H_2O_2 per minute at pH 7.0 and 25 °C. CAT specific activity was expressed as units of enzyme activity per milligram of total soluble protein (U mg^{-1}).

2.3.3. Determination of minimum inhibitory concentrations of PQ and H_2O_2

Yeast cells (previously grown on SDA medium for 48 h at 37 °C) were pre-cultured during 18 h at 37 °C and 120 rpm in Erlenmeyer flasks filled with 50 mL of SDB medium. Cellular concentration was quantified by cell counting and cells were harvested by centrifugation (3000 x *g*, for 10 min at 4° C) and re-suspended in 15 mL of RPMI medium in order to achieve a final cellular concentration of $1x10^6$ cells mL^{-1}. The cell suspensions of *Candida* species were treated with a range of concentrations of PQ and H_2O_2 (20, 50, 100, 150 and 200 mM, prepared in PBS). In each well of a 96-well microplate, 270 µL of each cell suspension were combined with 30 µL of the respective oxidant agent 10 times concentrated. Sterility controls (only RPMI medium) and growth controls (cellular suspension without chemical oxidants) were also included. These controls were also prepared in parallel in another 96-well plate to enable the measurement of absorbance in the beginning without compromising the sterility of the first microplate. After 24 h of incubation with oxidant agents (at 37 °C and 120 rpm), the absorbance was measured at 620 nm in an ELISA spectrophotometer (Rainbow, Tecan).

The cytotoxic effects of PQ and H_2O_2 were estimated as growth inhibition percentage (calculated in reference to the growth of untreated control cells) and expressed as Minimum Inhibitory Concentration - MIC_{90} - the lowest concentration of oxidant agent required to inhibit the growth of 90 % of organisms [232].

RPMI-1640 medium was prepared by dissolving 10.4 g L^{-1} of this powder in ultrapure water and adding 2 g L^{-1} of sodium bicarbonate and 34.53 g L^{-1} of MOPS (Sigma Aldrich). pH was adjusted to 7.0 and the medium was sterilized by filtration through a 0.22 µm cellulose filter and stored at 4 °C.

2.4. Statistical Analysis

Statistical analysis was performed using the statistical program GraphPad Prism 6. Data were evaluated using Student's t-test and one-way variance analysis (ANOVA) followed by Tukey's post-hoc test for multiple comparisons among experimental and control groups. The following p-values were considered: *p <0.05; **p <0.01; ***p <0.001; ****p <0.0001.

3. CHAPTER III

RESULTS AND DISCUSSION

3.1. Antimicrobial photodynamic therapy

The benzo[a]phenoxazinium chloride FSc was previously described as a potential photosensitizer for APDT of *C. albicans* planktonic cells [233] and *C. albicans* biofilms [227]. In the present study, the antifungal photodynamic effect of FSc against *C. glabrata* and *C. parapsilosis* biofilms was evaluated. For this purpose, the dark toxicity of FSc on *C. glabrata* and *C. parapsilosis* biofilms was firstly analyzed. The results of cellular growth after incubation of both *Candida* spp. biofilms with a range of FSc concentrations varying from 5 μM to 200 μM for 18 h are shown in Figure 7. According to Lopes *et al*. [227], an incubation time of 18 h was necessary to ensure the FSc uptake by extracellular matrix and cells of *C. albicans* biofilms.

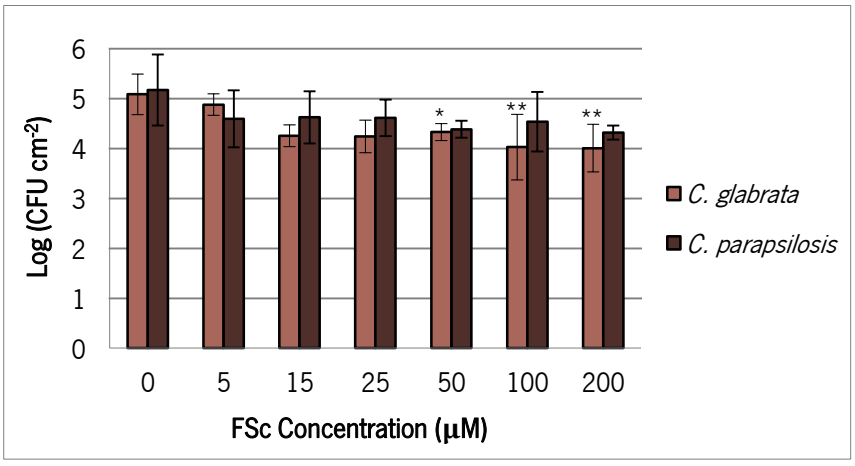

Figure 7. Logarithm of the number of *C. glabrata* ATCC 2001 and *C. parapsilosis* ATCC 22019 biofilm cells per cm^2 after 18 h of dark incubation with different concentrations of FSc. Values are average ± standard deviation of at least four independent experiments. * and ** indicates $p < 0.05$ and $p < 0.01$, and consequently statistically different from the respective control, 0 μM.

There was a significant decrease on *C. glabrata* biofilm viability for FSc concentrations of 50 μM ($p < 0.05$), 100 and 200 μM ($p < 0.01$). The inactivation effect at these concentrations was, respectively, 0.76 log, 1.06 log and 1.08 log CFU cm^{-2}. However, in biofilms of *C. parapsilosis*, no significant cell inactivation was obtained for experimental conditions tested. The resistance of *C. parapsilosis* can be explained by the extent of biofilm formation. Previous studies have demonstrated that *C. parapsilosis* biofilms have more total biomass than *C. glabrata* biofilms [53, 54]. Furthermore, it is known that *C. glabrata* forms a thin and compact biofilm consisting of yeast cells embedded within an extracellular matrix, while *C. parapsilosis* produces a compact biofilm, composed of both yeasts and pseudohyphae, with multilayers covering the entire surface of the substratum [54, 234]. These factors have a direct effect on the drug diffusion through biofilms and, consequently, in the final cell

inactivation. In fact, Al-Fattani and Douglas [235] have already demonstrated that the rates of drug diffusion through biofilms of *C. glabrata* are faster than those observed for biofilms of *C. parapsilosis*. In addition, the difference in the genetic determinants inherent to each *Candida* species may also influence the dye absorption and the consequent cellular response [201].

The analysis of dark toxicity was important to select the FSc concentrations to be used in APDT assays, because this therapy requires the use of a PS concentration below the values for which the compound present antifungal activity. On APDT assays, after dark incubation with FSc for 18 h, biofilms were irradiated with different light fluences, in order to optimize the light dose for maximal cells inactivation. The effects of light fluence and FSc concentration were studied.

Three FSc concentrations were selected for photodynamic treatment against *C. parapsilosis* biofilms - 50 μM, 300 μM and 500 μM. Biofilms treated with a higher concentration of FSc were subjected to a higher light dose during irradiation. The susceptibility of *C. parapsilosis* biofilms to the APDT assays using different concentrations of FSc and light fluences is shown in Figure 8.

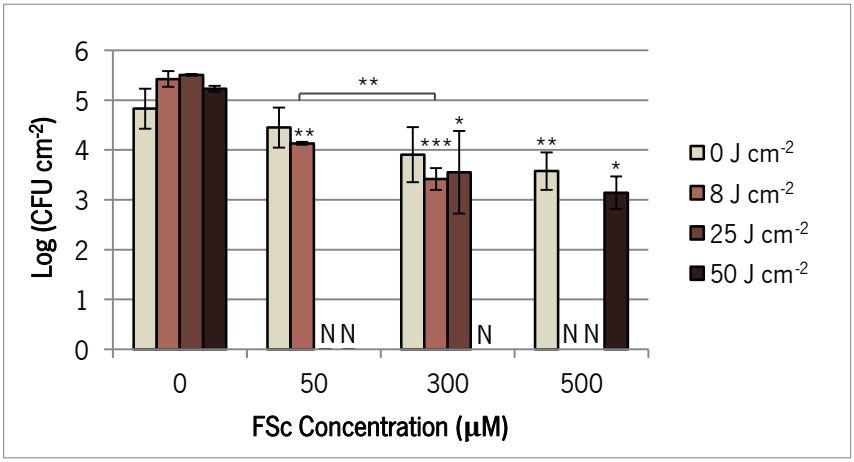

Figure 8. Logarithm of the number of *C. parapsilosis* ATCC 22019 biofilm cells per cm^2 after 18 h of dark incubation with different concentrations of FSc followed by irradiation with various light doses. Values are average ± standard deviation of at least two independent experiments. N – Not determined. *, ** and *** indicates $p < 0.05$, $p < 0.01$ and $p < 0.001$, and consequently statistically different from the control, 0 μM (at the same light fluence).

Light fluence *per se* (in the absence of dye) had no effect on *C. parapsilosis* biofilms viability. Such results were expected, since irradiation should not be harmful. The effect of dark incubation with FSc on cell viability slightly increases with dye concentration, but the differences were not statistically significant up to 300 μM. Only, at 500 μM of FSc, a significant 1.25 log cell inactivation was observed ($p < 0.01$) compared with the control. When compared to the respective control at 0 μM, the APDT

assays performed with an FSc concentration of 50 μM and 300 μM, at a light fluence of 8 J cm^{-2} showed a significant decrease of biofilm viability, with a reduction of 1.29 log ($p < 0.01$) and 2.01 log ($p < 0.001$), respectively. In addition, the difference observed between these two values is statistically significant ($p < 0.01$). Similarly, a significant ($p < 0.05$) decrease of cell viability (1.95 log) was achieved after APDT assays using 300 μM of FSc and a light fluence of 25 J cm^{-2}, in comparison with the control at 0 μM. The incubation with 500 μM of FSc and irradiation at 50 J cm^{-2} also resulted in a significant decrease ($p < 0.05$) of cell viability of 2.09 log, when compared with the control at 0 μM.

The decrease of cell viability described above was only dependent on the concentration of FSc. The killing effect of APDT was not dependent on the light dose delivered, since no significant differences were observed between biofilms incubated with FSc in the dark and those that were irradiated with increasing light doses. These results are not in agreement with previous studies, performed with planktonic cultures [236, 237] or biofilms [238] of *Candida* spp., in which APDT was able to kill cells in a fluence-dependent manner.

The susceptibility of *C. glabrata* biofilms to APDT is shown in Figure 9. Although there was a significant decrease on *C. glabrata* biofilm viability after incubation with 50 μM of FSc, the dark toxicity of the dye at this concentration was still very low. On the other hand, at 100 μM it was observed a higher dark toxicity of FSc. Thus, on APDT assays, biofilms were treated with an intermediate concentration of 75 μM of FSc. Given that the illumination in the absence of dye had no effect on *C. parapsilosis* biofilms viability, it was decided not to include the fluences of 8 and 25 J cm^{-2} at 0 μM in the controls. Thus, to draw conclusions about the effect of light fluence alone, the biofilms were irradiated with the highest light fluence.

As expected, light fluence *per se* had no effect on *C. glabrata* biofilm viability (in the absence of FSc). When compared with the control at 0 μM, biofilms incubated with 75 μM of FSc in the dark showed a significant ($p < 0.05$) decrease of cell viability (1.20 log). Likewise, it was observed a significant ($p < 0.01$) cell inactivation (2.11 log) after treatment with 75 μM of FSc and irradiation with a light fluence of 50 J cm^{-2} (compared with the control at 0 μM). Thus, once again, the antimicrobial activity of PDT was photosensitizer concentration-dependent. However, in this assay, APDT also produced a fluence-dependent loss of cell viability: biofilms treated with 75 μM of FSc and irradiated at a fluence of 50 J cm^{-2} showed a significant decrease of cell viability when compared with biofilms subjected to

FSc treatment in the dark (1.46 log; p <0.01) and biofilms treated with FSc at 8 J cm^{-2} (0.97 log; p <0.05).

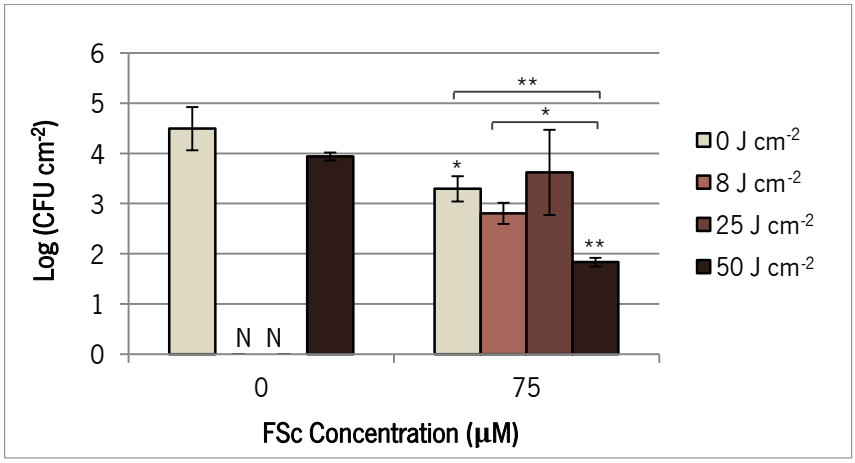

Figure 9. Logarithm of the number of *C. glabrata* ATCC 2001 biofilm cells per cm^{2} after 18 h of dark incubation with 75 µM of FSc followed by irradiation with various light doses. Values are average ± standard deviation of at least two independent experiments. N – Not determined. * and ** indicates p <0.05 and p <0.01, and consequently statistically different from the control, 0 µM (at the same light fluence).

Although photodynamic inactivation of biofilm cells was dependent on the light dose, the decline in cell viability was still low when compared to a previous study performed by Lopes *et al.* [227] (with the same irradiation system) in which a total inhibition of *C. albicans* biofilms was observed. Thus, a new assay was performed under the same conditions that led, in the aforementioned study, to eradication of *C. albicans* biofilms after incubation with 300 µM of FSc and illumination at a fluence of 36 J cm^{2} (Figure 10). In addition, biofilms were also irradiated with two intermediate light doses – 18 and 25 J cm^{2} – similarly to the other species' assays.

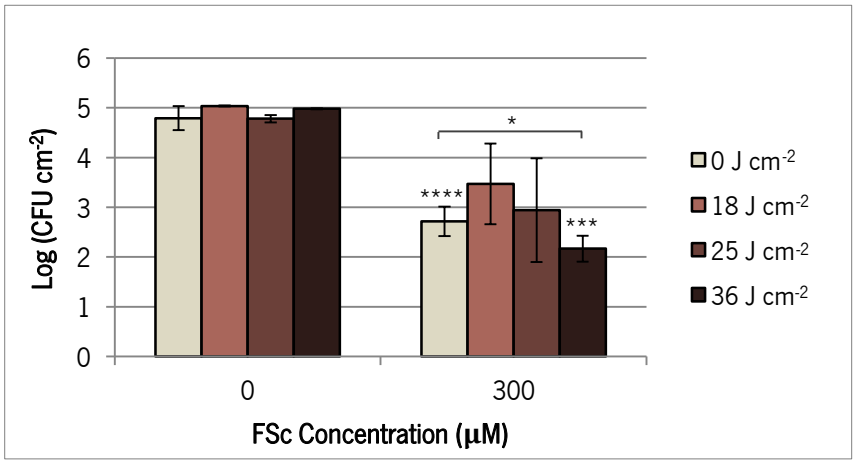

Figure 10. Logarithm of the number of *C. albicans* ATCC 90028 biofilm cells per cm^{2} after 18 h of dark incubation with 300 µM of FSc followed by irradiation with various light doses. Values are average ± standard deviation of at least two independent experiments. * indicates p <0.05; *** and **** indicates p <0.001 and p <0.0001, and consequently statistically different from the control, 0 µM (at the same light fluence).

As in previous assays, no effect of irradiation was observed in the absence of FSc. On the other hand, the dark incubation of *C. albicans* biofilms with 300 μM of FSc led to a significant ($p < 0.0001$) cell viability reduction of 2.07 log. *C. albicans* biofilms seem to be more sensitive to FSc than *C. parapsilosis* biofilms, which had only 1.25 log of cell inactivation at 500 μM of FSc. The structure of *C. albicans* biofilms involves two different layers: a thin, basal yeast layer and a thicker, less compact hyphal layer [239]. On the other hand, *C. parapsilosis* strains form quantitatively less and structurally less complex biofilms than *C. albicans* [240, 241]. Despite its less complex structure, previous studies have demonstrated that *C. parapsilosis* biofilms are similarly or even more resistant than *C. albicans* biofilms to conventional antifungals, such as amphotericin B and azole compounds [242–244].

Photodynamic inactivation was again photosensitizer concentration- and fluence-dependent. The incubation of biofilms with 300 μM of FSc and irradiation with a light fluence of 36 J cm^{-2} resulted in a significant ($p < 0.001$) cell inactivation (2.81 log reduction), when compared with the control at 0 μM. At the same time, it was observed a significant ($p < 0.05$) decrease of cell viability (0.55 log) between these biofilms and those that were incubated in the dark with 300 μM of FSc. However, cell inactivation was much less pronounced than that observed by Lopes *et al.* [227] (that showed a total inhibition of biofilm viability). Once dark toxicity of FSc at 300 μM was similar in both studies (with a cell viability reduction of 2.1 log), it is possible to conclude that these differences are only related with the irradiation of biofilms. As the aim was to use the same irradiation conditions (light power, irradiated area and irradiation time), and no adjustments were made between the experiments, the differences in the results could be explained by the sensitivity of the irradiation system. This factor may also explain the low inactivation of *C. parapsilosis* and *C. glabrata* biofilms after irradiation with increasing light doses.

C. albicans biofilms were also subjected to APDT tests with two widely used PSs – Nile blue chloride and PpIX - in order to compare the efficiency of APDT in their presence with FSc-mediated APDT assays. Figure 11 presents the cell viability after incubation with 300 μM of each PS and irradiation with a light fluence of 36 J cm^{-2}.

It seems that Nile blue had some dark activity, with a significant ($p < 0.001$) cell viability reduction of 1.56 log, in contrast to PpIX that had no dark activity against *C. albicans* biofilms. This can be

explained by the size of both dyes: Nile blue is smaller than PpIX and therefore can more easily penetrate the inner layers of the biofilm and promote a better sensitization of the cells [110]. It is interesting to note that the reduction of cell viability obtained with Nile blue was similar to that achieved with FSc. This can be a result of their structural similarities, since Nile blue is a benzophenoxazine dye like FSc.

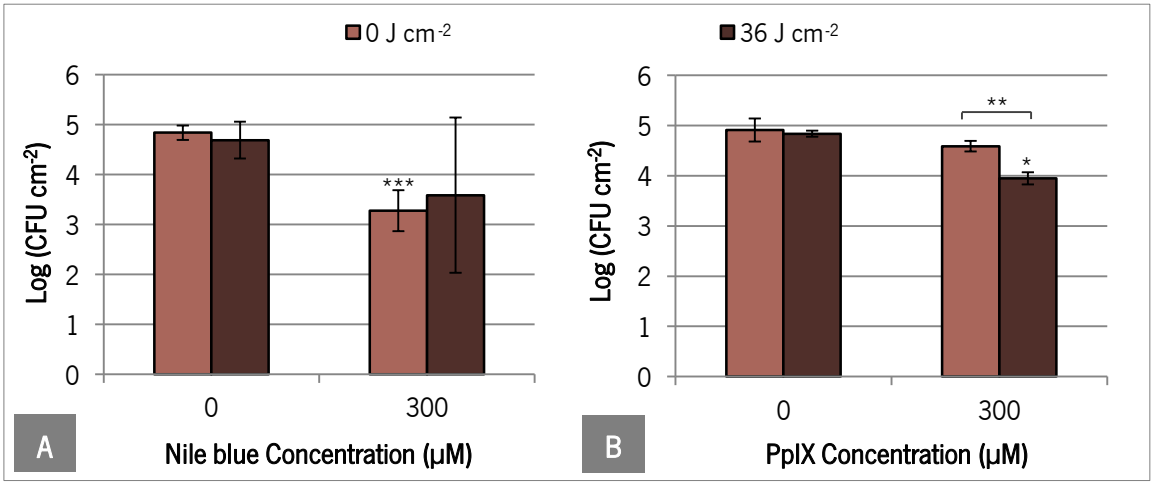

Figure 11. Logarithm of the number of *C. albicans* ATCC 90028 biofilm cells per cm^2 after 18 h of dark incubation with 300 μM of Nile blue chloride **(A)** and PpIX **(B)** followed by irradiation with a light dose of 36 J cm^{-2}. Values are average ± standard deviation of at least two independent experiments. ** indicates $p < 0.01$; * and *** indicates $p < 0.05$ and $p < 0.001$, and consequently statistically different from the control, 0 μM (at the same light fluence).

Nile blue-mediated APDT assays showed no significant cell inactivation of *C. albicans* biofilms. The results were not conclusive, however, it is known that Nile blue has a low photosensitizing ability [245–248]. The use of Nile blue derivatives (obtained by suitable structural modifications) with improved singlet oxygen quantum yield could help to overcome this problem [247, 249]. It has already been shown that some Nile blue chalcogen analogues, including derivative 5-(ethylamino)-9-diethylamino-benzo[a]phenothiazinium chloride (EtNBS) and 5-(ethylamino)-9-diethylamino-benzo[a]phenoselenazinium chloride (EtNBSe), can have a great photo-induced activity against *C. albicans*, *E. coli* and *Enterococcus faecalis* [137]. The positive charge of EtNBS suggests that this Nile blue derivative can mediate the photodestruction of a wide range of microorganisms, despite the fact that this particular compound has a relatively low singlet oxygen quantum yield [250, 251]. EtNBS- and EtNBSe-mediated APDT have also been used against Leishmaniasis parasites *in vitro* [163].

A significant ($p < 0.05$) cell inactivation of 0.89 log was reached after incubation with 300 μM of PpIX and irradiation with 36 J cm^{-2}, in comparison with the control at 0 μM. For the same irradiation conditions, a higher inactivation of *C. albicans* biofilms was obtained with 300 μM of FSc (2.81 log) than with 300 μM of PpIX. This was to be expected, given the difference in size between the two dyes. PpIX is larger than FSc and therefore has greater difficulty than FSc in penetrating the biofilm. At the same time, it was observed a significant ($p < 0.01$) cell inactivation (0.64 log) between biofilms treated in the dark with PpIX and those that were subjected to APDT. Therefore, as in previous assays, microbial photoinactivation occurred in a photosensitizer concentration- and fluence- dependent manner.

To date, no studies evaluating the effect of exogenous PpIX-mediated APDT against *Candida* spp. can be found in the literature. The majority of published studies have been realized with ALA or its derivatives. The ability of ALA-induced PpIX to kill *C. albicans* suspensions upon irradiation has been demonstrated *in vitro*. Monfrecola *et al.* [183] achieved a complete inactivation of *C. albicans* after dark incubation with 600 mg mL^{-1} of ALA for 3 h and irradiation with a light dose of 40 J cm^{-2}. Donnelly *et al*. [252] also reported a reduction in viability of 90 % after incubation of *C. albicans* with 100 mM of ALA for 30 min followed by irradiation with 100 J cm^{-2} of red light. Nevertheless, some authors have reported the high effectiveness of exogenous PpIX-mediated APDT against bacteria [163, 253, 254]. Grinholc *et al*. [254] have demonstrated that exogenous PpIX can exert an effective antibacterial activity against methicillin-resistant *Staphylococcus aureus* strains after incubation with solely 10 μM of PpIX for 15 min (in the dark) and illumination with a light dose of only 12 J cm^{-2}. Furthermore, the authors demonstrated that exogenous PpIX-mediated APDT, which induced a 2.4 log reduction in viable counts, was considerably more effective than photodynamic treatment with ALA-induced endogenous PpIX, which only caused a 1.6 log reduction in viable counts, even after 24 h of incubation with 10 mM of ALA and irradiation with a light dose of 50 J cm^{-2}. Exogenous PpIX-mediated APDT was also studied by Awad *et al*. [253]. In their study, incubation of planktonic cells of *Staphylococcus aureus* with 30 μM of PpIX for 15 min, followed by illumination with a light dose of 18 J cm^{-2} yielded a 1.5 log decrease in the number of viable cells.

In the present study, after irradiation, biofilms were immediately scraped to proceed with CFUs count. An increase in the time of cells exposure to irradiation medium could result in a better response of APDT assays. In fact, Rosseti *et al*. [255] observed that the effects of APDT are amplified when cells are maintained in the medium of irradiation for more prolonged times, indicating that species

produced by APDT induce continuous effects on cells. An increase of ROS concentration and cell permeability with the incubation time was observed. Rosseti *et al.* [255] also studied the effect of APDT combined with 0.1 mg mL^{-1} of TB on inhibition of cell growth and biofilm formation. Cell growth inhibition was approximately 52 %, 73 % and 82 %, in cells maintained in the irradiation medium for 1 h, 2 h and 3 h, respectively. The reduction of biofilm formation was 30 %, 50 % and 62 %, in cells submitted to incubation times of 1 h, 2 h and 3 h, respectively. These observations emphasize the potential of APDT to reduce both cell proliferation and colonization.

Efficient photodynamic inactivation of *Candida* spp. biofilms has been demonstrated for several authors. Pereira *et al.* [256] found a reduction of 2.32 log CFU of *C. albicans* biofilms after incubation with MB (0.1 mg mL^{-1}) for 5 min and irradiation with an energy density of 350 J cm^{-2}, using a InGaAlP (indium–gallium–aluminum–phosphorus) laser light. Donnelly *et al.* [198] achieved higher death levels of *C. albicans* biofilms, with a log reduction of 1.5 to 6.5, after treatment with 5 mg mL^{-1} of TBO during 30 min, followed by irradiation with an energy density of 200 J cm^{-2}. The treatment of *Candida* biofilms with 1 μM of XF-73 (5,15-bis-[4-(3-trimethylammoniopropyloxy)-phenyl]-porphyrin) or 50 μM of TMPyP (5,10,15,20-tetrakis(1-methyl-4-pyridyl)-21H,23H-porphine, tetra-p-tosylate salt) - two and four-fold positively charged porphyrins - during 4 h, followed by illumination with a light fluence of 48.2 J cm^{-2}, also caused a significant decrease of biofilm viability, with a reduction of 5 log [257]. The conjugation of gold nanoparticles (GNP) with PS was previously reported as an efficient method for photodynamic inactivation of *C. albicans* biofilms. Khan *et al.* [258] found a reduction of *Candida* biofilm of 95.4 % by GNP–MB conjugate, as compared to MB alone, which reduced the biofilm by 81.9 %. Although some studies reported the susceptibility of *Candida* biofilms to APDT, few studies have observed its complete inactivation. Beirão *et al.* [259] used a cationic porphyrin - Tetra-Py^{+}-Me (5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide) - at 5 μM for 30 min, combined with irradiation at 43.2 J cm^{-2} and observed the completely inactivation (6.5 log) of *C. albicans* biofilms.

APDT can also suppress the pathogenicity of *Candida* spp. due to inhibition of its adhesion, one of the prerequisites for *Candida* successful colonization and posterior infection, possibly through the effects on the cytoplasmic membrane. Soares *et al.* [260] demonstrated that APDT with a light dose of 180 J cm^{-2} combined with 25 μM TBO inhibited 55 % of the adhesion of the *Candida* strains to buccal epithelial cells.

3.2. Oxidative stress response

Once oxidative stress is the leading cause of cell death in cells treated with APDT, the study of oxidative stress response of *Candida* species is extremely important. In this study, the effect of two oxidative stress inductors, H_2O_2 and PQ, on both cell viability and specific activities of antioxidant enzymes (CAT and SOD) was analyzed. To induce an oxidative stress response of the species *C. albicans*, *C. glabrata* and *C. parapsilosis*, cellular suspensions were treated with 2 mM of PQ or H_2O_2 during 3 h (Figure 12).

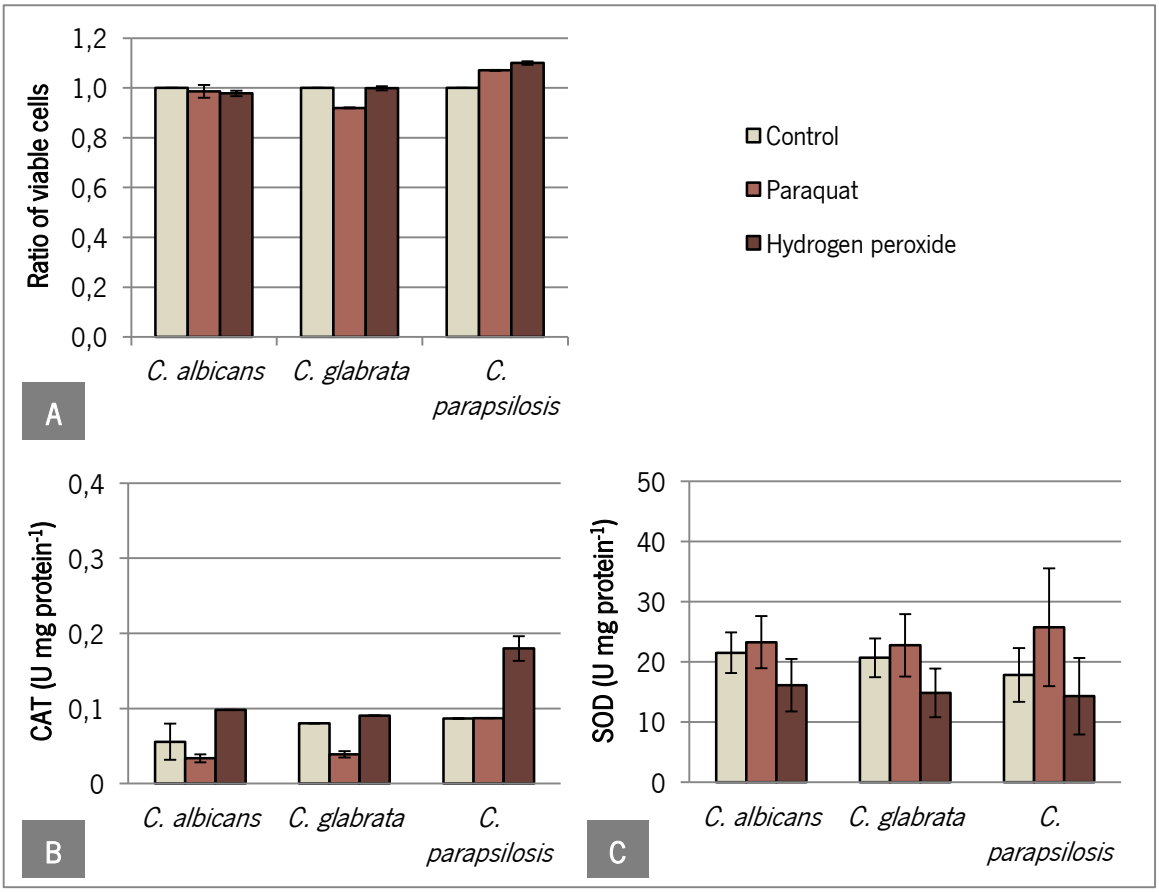

Figure 12. Ratio of viable cells (ratio of cell viability between each treatment and the respective control) **(A)**; CAT specific activity **(B)** and SOD specific activity **(C)** of *C. albicans* ATCC 90028, *C. glabrata* ATCC 2001 and *C. parapsilosis* ATCC 22019 exposed to 2 mM of PQ or H_2O_2 for 3 h. Values are average ± standard deviation of at least three replicate measurements.

A slight decrease of *C. glabrata* viability was observed after incubation with PQ (Figure 12.A). These results suggest that *C. glabrata* is more susceptible to PQ than the other species, since there was no cell viability loss of *C. albicans* and *C. parapsilosis* suspensions after treatment with this oxidant agent. When *C. albicans, C. glabrata* and *C. parapsilosis* were exposed to H_2O_2, no changes in cell viability were observed, which shows that these species are resistant to this concentration of H_2O_2.

These results are in agreement with Kaloriti *et al.* [261], which observed that *C. albicans* and *C. glabrata* are relatively resistant to 5 mM and 10 mM of H_2O_2, respectively.

The resistance of *Candida* species to H_2O_2 can be explained by the increased levels of CAT specific activity observed in the presence of this oxidant agent (Figure 12.B). As expected, a higher CAT induction was observed in yeast cells exposed to H_2O_2 than those exposed to PQ. A 2.9-, 2.3- and 2.1-fold enhancement on CAT induction was obtained in *C. albicans, C. glabrata* and *C. parapsilosis* treated with H_2O_2, respectively, compared to the experiments conducted with PQ. However, when compared with the control, *C. parapsilosis* showed a higher CAT induction (2.1-fold), followed by *C. albicans* (1.8-fold) and *C. glabrata* (1.1-fold). Other studies have demonstrated an increase in CAT activity as a result of exposure of *Candida* spp. to H_2O_2 [262–265]. It is interesting to note that, as previously observed by other authors [263], in the presence of H_2O_2, *C. parapsilosis* has a higher CAT activity than *C. albicans* and *C. glabrata*.

Although there were no significant differences in SOD enzyme activity among the experiments with PQ or H_2O_2, it is possible to observe that *Candida* cells responded to PQ by increasing their content of SOD comparatively to the control (Figure 12.C). The same result was observed by other researchers in their experiments with *E. coli*, *Yarrowia lipolytica* and *Pichia pastoris* [266, 267].

In order to evaluate the antioxidant response induced by a higher concentration of ROS-inducing agents, cellular suspensions of *C. albicans, C. glabrata* and *C. parapsilosis* were incubated with 20 mM of PQ or H_2O_2 during 3 h (Figure 13). There was a significant reduction of *C. glabrata* viability after its exposure to 20 mM of PQ (Figure 13.A). However, when cells of *C. albicans* and *C. parapsilosis* were exposed to the same concentration of PQ, no changes in cell viability were observed. It seems that *C. glabrata* is more sensitive to PQ than the other species, despite the SOD activity of this yeast be slightly higher than those obtained in *C. albicans* and *C. parapsilosis* cultures (Figure 13.C). In the experiments with H_2O_2, there were no significant differences on the cellular viability, which shows that all species are quite resistant to this oxidant agent concentration. In fact, other authors have demonstrated that cells of *C. albicans* and *C. glabrata* are resistant to 20 mM of H_2O_2 under specific conditions [265, 268, 269].

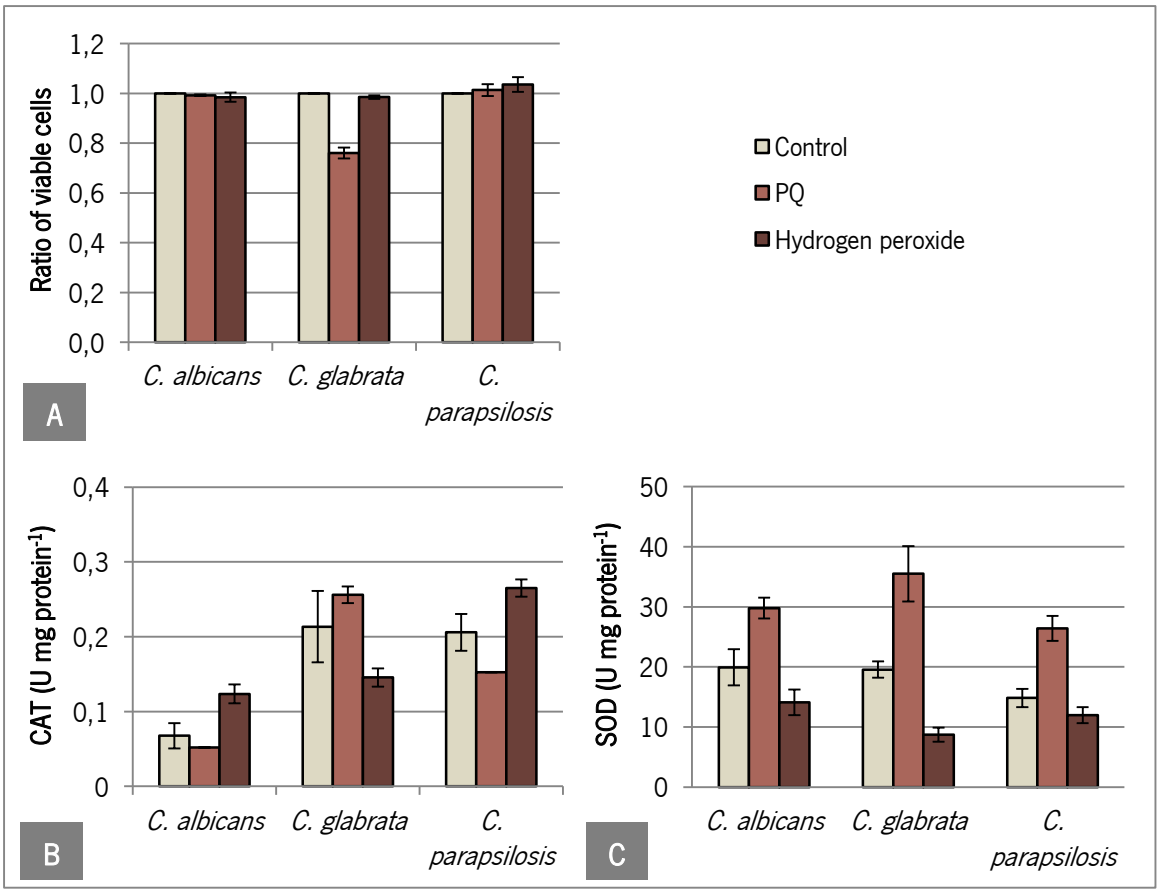

Figure 13. Ratio of viable cells (ratio of cell viability between each treatment and the respective control) **(A)**; CAT specific activity **(B)** and SOD specific activity **(C)** of *C. albicans* ATCC 90028, *C. glabrata* ATCC 2001 and *C. parapsilosis* ATCC 22019 exposed to 20 mM of PQ or H_2O_2 for 3 h. Values are average ± standard deviation of at least three replicate measurements.

The exposure of *C. albicans* and *C. parapsilosis* to H_2O_2 led to a 2.4- and 1.7-fold increase in CAT activity, respectively, compared to the experiments performed with PQ (Figure 13.B). A 1.8- and 1.3-fold improvement in CAT activity was observed in *C. albicans* and *C. parapsilosis* cells, respectively, compared to the control. This factor contributes, at least in part, to the higher cell viability of both species. Nevertheless, *C. glabrata* cells exhibit a contradictory behavior, with a greater induction of CAT after treatment with PQ, instead of H_2O_2.

As expected, an increase in total SOD activity was observed after exposure of *Candida* species to PQ (Figure 13.C). Compared to the control (without exposure to PQ), *C. glabrata* and *C. parapsilosis* showed a similar SOD induction of 1.8-fold, which was slightly greater than those of *C. albicans* (1.5-fold). The SOD content of *C. albicans, C. glabrata* and *C. parapsilosis* after treatment with PQ was higher (2.1-, 4.1- and 2.2-fold, respectively) than the value obtained in experiments with H_2O_2. As previously reported by Lopes *et al.* [267], the low SOD activity in cells incubated with H_2O_2 suggests

that this enzyme does not participate in the cellular defense mechanisms against this oxidant agent. In fact, Abbeg *et al.* [263] have already showed that the incubation of *Candida* strains with H_2O_2 only caused a small induction of SOD.

It is important to note that the treatment of cells with 20 mM of PQ allows a greater induction of SOD when compared with the previous test, performed with a lower concentration of this oxidant agent. Furthermore, SOD activity was substantially higher than CAT activity in both assays (Figure 12 and Figure 13), suggesting that SOD plays a major role on antioxidant defense against the agents tested. In fact, this enzyme is one of the primarily induced antioxidant enzymes involved in oxidative stress response [267].

In order to understand the effect of PQ and H_2O_2 on the viability of *C. albicans*, *C. glabrata* and *C. parapsilosis*, the percentage of growth inhibition of these *Candida* species after 24 h of treatment with different concentrations of the two oxidant agents was determined (Figure 14). MIC_{90} of PQ and H_2O_2 for the *Candida* species are presented in Table 5.

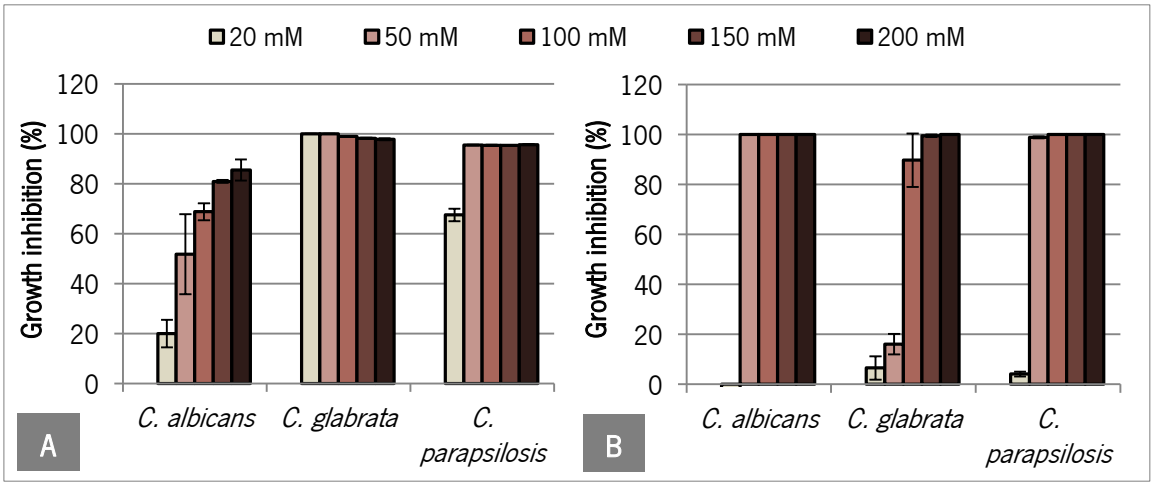

Figure 14. Growth inhibition (%) of cell suspensions of *C. albicans* ATCC 90028, *C. glabrata* ATCC 2001 and *C. parapsilosis* ATCC 22019 (adjusted to 10^6 cells mL^{-1}), after incubation with different concentrations of PQ **(A)** and H_2O_2 **(B)** during 24 h. Values are average ± standard deviation of two independent experiments.

The results indicate that *C. albicans* was more resistant to PQ than *C. parapsilosis* and *C. glabrata*. This last one was the most sensitive specie to PQ, with a complete growth inhibition after treatment with 20 mM of this oxidant agent (Figure 14.A). Other authors have reported the sensitivity of *C. glabrata* to high concentrations of superoxide anion, in contrast to *C. albicans,* which is more resistant

[268, 270]. These studies were performed with menadione, a redox cycling agent capable of generating intracellular superoxide anions, like PQ.

Table 5. MIC_{90} of PQ and H_2O_2 (mM) of *C. albicans* ATCC 90028, *C. glabrata* ATCC 2001 and *C. parapsilosis* ATCC 22019.

	C. albicans	*C. glabrata*	*C. parapsilosis*
PQ	> 200	0 – 20	20 – 50
H_2O_2	20 – 50	50 – 100	20 – 50

Despite being the most sensitive specie to PQ, *C. glabrata* was quite resistant to H_2O_2, when compared to the other species. In fact, previous studies have shown that cells of *C. glabrata* are more resistant to H_2O_2 than those of *C. albicans* [261, 268, 269, 271]. Cuéllar-Cruz *et al*. [271] also reported that stationary-phase cells of *C. glabrata* are extremely resistant to high levels of H_2O_2, up to 1 M, compared to about 300 mM of H_2O_2 for *C. albicans.* The results suggest that *C. parapsilosis* is even more sensitive to H_2O_2 than *C. albicans*, showing a small growth inhibition after incubation with 20 mM of H_2O_2 (Figure 14.B). Abegg *et al*. [263] also noted an increased sensitivity of *C. parapsilosis* to this oxidant agent, compared with *C. albicans.* The resistance of *C. albicans* to H_2O_2 has been related with the presence of trehalose, a disaccharide that acts synergistically with the antioxidant enzymes, by quenching oxygen radicals, and protects cell integrity from ROS [264, 272].

4. CHAPTER IV

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Despite all the progress that has been made in APDT, in particular regarding the development of new PSs, the optimization of existing protocols or the development of new procedures for the treatment of different types of fungal infections are required. Therefore, the first goal of this work was to evaluate and optimize the activity of FSc-mediated APDT against biofilms of *C. glabrata, C. parapsilosis* and *C. albicans.* The efficiency of photodynamic inactivation by Nile blue chloride and PpIX on *C. albicans* biofilms was also evaluated. Regarding the dark toxicity of FSc, *C. glabrata* biofilms showed to be more sensitive than *C. albicans* and *C. parapsilosis.* This last one showed a high level of resistance to FSc. While PpIX showed no dark activity against *C. albicans* biofilms, Nile blue chloride demonstrated a great dark toxicity. Concerning to the APDT assays, only FSc and PpIX exhibited a significant inactivation of *Candida* biofilms. Photodynamic inactivation proved to be dye concentration- and light fluence-dependent. In this study, the irradiation of biofilms with increasing light doses only resulted in a slight decrease of biofilm viability.

In conclusion, this work contributed to enlarge the knowledge about the action of APDT mediated by FSc, Nile blue chloride and PpIX on *Candida* biofilms. The results obtained after APDT of *C. albicans* biofilms mediated by FSc were quite different from those observed in a previous work [227]. This shows the complexity of these studies due to variations that may occur in the chemical or physical agents involved in the photodynamic process (e.g. light source). Thus, additional studies would be needed to understand these uncertainties. Moreover, it would be really useful to clarify the interactions of FSc on a cellular level. The determination of its subcellular targets will help to understand the photosensitizing activity of FSc and the mechanisms of cell death. Another interesting approach will be to optimize the irradiation system, in order to ensure light delivery in a homogeneous and non-divergent manner. In the experiments with Nile blue no conclusive results were obtained, therefore it would be also interesting to repeat these assays.

Only few studies reported the photodynamic activity with the exogenous dye PpIX, and almost refer to bacteria. Thus, this work had an important role in an initial approach of the antifungal activity of exogenous PpIX against *Candida* spp. Nevertheless, it would be important to perform new studies in order to determine the appropriate parameters - dye concentration, pre-irradiation time, and light dose - for maximum PpIX-mediated APDT effectiveness.

Microorganisms in localized infections commonly exist in the form of dual biofilms, and the association between *C. albicans* and *C. glabrata* is a stark example of the dual species *Candida*

biofilms. Thus, evaluation of the activity of FSc and PpIX-mediated APDT against these biofilms could also be an interesting field to explore. Furthermore, it would be interesting to extend the study of photodynamic inactivation mediated by FSc and PpIX to clinical isolates of *Candida* species, and evaluate the cytotoxic effects on mammalian cells arising from the incubation with these dyes, aiming the development of new protocols for application of APDT on clinical practice, which is the main objective of all the studies performed in this field.

Another important part of this work aimed to characterize the oxidative stress response of *Candida* spp. to H_2O_2 and PQ. The results suggested that *Candida* species have different levels of resistance to oxidant agents and they use different defense mechanisms against these substances, showing differences in the induction of antioxidant enzymes. Nevertheless, it was observed that cell responses against both peroxide and superoxide stress agents comprise enhanced expression of CAT and SOD, respectively.

This work also provided a significant increase of knowledge about the cellular response of *Candida* species to oxidative stress. As future work, it would be appropriate to evaluate the role of other important antioxidant enzymes on the cell defense mechanisms. Moreover, it would be interesting to extend this approach to the *Candida* biofilms and to assess the differences in the cellular response of these biofilms and those undergoing APDT. This would be useful to understand the difference between chemical and APDT treatment.

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