**Universidade de Aveiro** Departamento de Ciências Médicas

**Ano 2019**

**Vera Catarina Ferreira de Sousa**

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# Fotossensibilizadores porfirínicos catiónicos na inativação de *Candida albicans* em sangue

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em (Mestrado de Biomedicina Molecular), realizada sob a orientação científica da Professora Doutora Maria Adelaide de Pinho Almeida, Professora Auxiliar com agregação do Departamento de Biologia da Universidade de Aveiro, e coorientação da Professora Doutora Maria do Amparo Ferreira Faustino, Professora Auxiliar do Departamento de Química da Universidade de Aveiro.

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Dedico este trabalho ao meu irmão e pais pelo apoio incondicional

**o júri**
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**palavras-chave**

Terapia fotodinâmica antimicrobiana, *Candida albicans*, porfirinas, azul de metileno, sangue, plasma sanguíneo, fotossensibilizador

**resumo**

O sangue é um recurso essencial, porém também é uma fonte de transmissão de infeções. Consequentemente, a capacidade de desinfetar o sangue e seus derivados assume uma grande importância na prática clínica. Atualmente, o método de desinfeção mais eficaz na inativação de microrganismos usado apenas em plasma e concentrados proteicos, baseia-se no uso combinado do tri(*n*-butil)fosfato e do detergente Tween 80. No entanto, estes produtos devem ser removidos após o procedimento de desinfeção, pois são prejudiciais quer para as membranas dos eritrócitos quer para as plaquetas dos indivíduos transfundidos. A Terapia Fotodinâmica Antimicrobiana (aPDT) tem sido sugerida como uma técnica alternativa à desinfeção do sangue. Os fotossensibilizadores (PS) azul-de-metileno (MB), derivados de psoraleno e riboflavina já se encontram aprovados em alguns países para desinfetar plasma e plaquetas, no entanto não há até à data nenhuma aplicação aprovada baseada em aPDT para a desinfeção de sangue total e de concentrados de eritrócitos. O objetivo deste estudo foi avaliar a eficácia da aPDT na inativação de *Candida albicans*, um microorganismo frequentemente envolvido em infeções sistémicas. Para isso, foram usados derivados porfirínicos catiónicos na fotoinativação de *C. albicans* em tampão fosfato-salino (PBS), plasma e sangue total. Uma vez que o MB é o PS mais usado para desinfetar plasma, a sua eficácia também foi avaliada para comparar com a eficiência dos derivados porfirínicos testados. As amostras e os controlos foram expostos durante 270 min a luz branca (380-700 nm) com uma irradiação de 2,5 e 150 mW/cm$^2$ para PBS e sangue total / plasma, respetivamente.

Todos os derivados porfirínicos catiónicos testados foram eficazes na fotoinativação de C. *albicans* em PBS, no entanto, o MB nas mesmas condições (concentração e dose de luz) não demonstrou possuir capacidade de inativar *C. albicans*. No plasma, a FORM e Tri-Py(+)-Me provaram ser fotossensibilizadores promissores na inativação de *C. albicans*. Apesar de no sangue total as taxas de fotoinativação obtidas com os fotossensibilizadores porfirínicos tenham sido mais altas que as obtidas com MB, mais estudos serão necessários para melhorar as taxas de inativação obtidas. No entanto, os resultados indicam que a aPDT usando fotossensibilizadores porfirínicos catiónicos parece ser uma abordagem promissora para a fotoinativação de *C. albicans* no plasma.

Destaca-se o facto que nenhum destes fotossensibilizadores porfirínicos nas concentrações testadas no plasma e no sangue total promoveu hemólise significativa em condições isotónicas. Uma situação similar foi observada quando foi avaliada a hemólise após a adição de plasma tratado com esses PSs a concentrados de eritrócitos.

**keywords**

Antimicrobial Photodynamic Therapy, *Candida albicans*, cationic porphyrins, methylene blue, blood plasma, whole blood, photosensitizer

**Abstract**

Blood is an essential body fluid but is yet a source of microbial infections transmission. Consequently, the ability to disinfect blood and its derivatives has assumed great importance. Currently, the most used method for inactivating microorganisms, which can be only used in plasma or protein concentrates, is the combined use of a tri(*n*-butyl)phosphate and the detergent Tween 80. However, these chemicals must be removed after disinfection procedure once they are harmful to the membranes of erythrocytes and platelets of blood receptors. Antimicrobial Photodynamic Therapy (aPDT) has been suggested as an alternative technique to blood disinfection. Methylene blue (MB), psoralen and riboflavin are already approved photosensitizers (PS) in some countries to disinfect plasma/platelets, but there is no aPDT approved application for whole blood and erythrocytes concentrates. The aim of this study was to evaluate the effectiveness of aPDT to inactivate *Candida albicans* in blood, a microorganism frequently involved in bloodstream infections. For that, several cationic porphyrin derivatives were used to photoinactivate *C. albicans* in phosphate buffered saline (PBS), plasma and whole blood. Once MB is the mostly used PS to disinfect plasma, its efficacy was also evaluated for comparison. Samples and controls were exposed for 270 min to white light (380-700 nm) at an irradiance of 2.5 $mW/cm^2$ and 150 $mW/cm^2$ for PBS and whole blood/plasma, respectively.

All the tested cationic porphyrin derivatives were effective to photoinactivate *C. albicans* in PBS. However, MB under the same conditions (concentration and light dose) did not appear to be efficient in *C. albicans* inactivation. In plasma, FORM and Tri-Py(+)-Me proved to be promising PSs in *C. albicans* photoinactivation. Although in whole blood the inactivation rates obtained with porphyrin derivatives were higher than the ones obtained with MB, further improvements are required. Nevertheless, the results indicate that aPDT using cationic porphyrinic photosensitizers seems to be a promising approach for the photoinactivation of *C. albicans* in plasma.

Additionally, none of these PSs, in the tested concentrations in plasma and whole blood, had promoted significant hemolysis at the isotonic conditions when hemolysis was evaluated in whole blood and after the addition of aPDT treated plasma with these PSs to concentrated erythrocytes.

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# List of abbreviations

| | |
|---|---|
| **aPDT** | Antimicrobial Photodynamic Therapy |
| **CFU** | Colony Forming Units |
| **DC** | Dark Control |
| **LC** | Light Control |
| **PS** | Photosensitizer |
| **Di-Py(+)-Me *adj*** | 5,15-bis(1-methylpyridinium-4-yl)-10,20 bis(pentafluorophenyl)porphyrin di-iodide |
| **Di-Py(+)-Me *opp*** | 5,10-bis(1-methylpyridinium-4-yl)-15,20-bis(pentafluorophenyl)-porphyrin di-iodide |
| **Mono-Py(+)-Me** | 5-(1-methylpyridinium-4-yl)-10,15,20-tris(pentafluorophenyl)-porphyrin iodide |
| **MB** | Methylene Blue |
| **TBO** | Toluidine Blue |
| **RB** | Rose Bengal |
| **Tetra-Py(+)-Me** | 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide |
| **Tri-Py(+)-Me** | 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin tri-iodide |
| **DMSO** | Dimethyl sulfoxide |
| **PNS** | 1H-Phenalen-1- one-2-sulfonic acid |
| **MRSA** | *Staphylococcus aureus* resistant to methicillin |
| **OH•** | Hydroxyl Radicals |
| **$O2^{\bullet -}$** | Superoxide Anions |
| **$^1O_2$** | Singlet oxygen |
| **$^3O_2$** | Molecular dioxygen |
| **ROS** | Reactive Oxygen Species |
| **TTI** | Transfusion-transmitted infections |
| **Rpm** | Rotations per minute |
| **YGCA** | Yeast Glucose Chloramphenicol Agar |

| | |
|---|---|
| **YG** | Yeast Glucose |
| **PBS** | Phosphate Buffered Saline solution |
| **HSCT** | Hematopoietic Stem Cells Transplants |
| **SOT** | Solid Organ Transplants |
| **Als** | Agglutinin-like sequence |
| **Saps** | Secreted Aspartic Proteins |
| **ALA** | Aminolevulinic Acid |

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## Chapter I

# Thesis outline

In this study it was intended to evaluate if aPDT with cationic porphyrinic photosensitizers can be an alternative to disinfect blood and blood products. Blood, despite to be an essential biological fluid, can be a main source of transmission of bacterial, viral and fungal infections. Currently three non-porphyrinic photosensitizers (Methylene Blue, Psoralen and Riboflavin) are already approved for the treatment of plasma and platelets, but no aPDT protocol is yet approved to treat the whole blood and erythrocyte concentrates.

This document is divided into three parts.

The first part (**Chapter I**), consists in a general introduction. In this section it was described the state of the art and the basic concepts about the photodynamic therapy approach, mechanism of action and photosensitizers in current use. Also in this chapter, it was discussed the disinfection methods of blood and blood products (plasma, protein concentrates and platelets). It was also referred the therapeutic strategies for the treatment of *C. albicans*, its susceptibility to antifungal agents as well as the development of resistance by this fungus and their main virulence factors.

The second part (**Chapter II**) refers to the study of *C. albicans* photoinactivation in blood plasma and whole blood using a cationic porphyrin derivative (Tri-Py(+)-Me) and a PS formulation (FORM), based on a non-separated mixture of five cationic *meso*-tetraarylporphyrin derivatives. This chapter corresponds to a manuscript already published where it is described the work performed during this study, the experimental setup, results and discussion showing that aPDT can represent an alternative to the conventional disinfection techniques of blood.

In this document it is also presented in **Appendix** another study in which I performed all the biological assessments and the discussion of the biological results. I participated also in the preparation of the manuscript which is in a final phase of submission. In this study, the efficiency of five neutral nitroindazole-porphyrin derivatives and their combined effect with KI in the photoinactivation of *S. aureus* resistant to methicillin (MRSA) was evaluated.

- **Manuscript already published:**

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- **Manuscript in submission phase:**

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- **Poster participations:**

Photodynamic inactivation of *Candida albicans* in blood

Vera Sousa, Ana T.P.C. Gomes, Maria A.F. Faustino, Maria G. P. M. S. Neves and Adelaide Almeida

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Photodynamic inactivation of *Candida albicans* in blood

Vera Sousa, Ana T.P.C. Gomes, Maria A.F. Faustino, Maria G. P. M. S. Neves and Adelaide Almeida

One health day, CESAM, University of Aveiro, 6th November

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# Chapter I- Introduction

## 1. Fungal infections

Fungi are more and more recognized as major pathogens in critically ill patients. A vast majority of infections in these patients are due to either *Candida* spp. (35%-91%) or *Aspergillus* spp. (9%-52%), with other opportunistic fungi accounting for 1-2% of the fungal infections. *Candida* spp. and *Cryptococcus* spp. are the yeasts most frequently isolated in clinical practice [1]. Although the most frequent filamentous fungi isolated are *Aspergillus* spp., the *Fusarium* spp., *Scedosporium* spp., *Penicillium* spp. and Zygomycetes are progressively seen.

### 1.1. Opportunistic fungi

In the past decade the frequency of opportunistic fungal infections has increased and the spectrum of these fungal pathogens has changed [2]. Various reasons have been suggested for the increase in the incidence of invasive fungal infections, including the use of immunosuppressive agents, broad-spectrum antimicrobials, prostheses, grafts and more aggressive surgeries. Patients with burns, neutropenia, human immunodeficiency viruses (HIV) and pancreatitis are additionally predisposed to fungal infections [3]. Patients with acute leukemia, hematopoietic stem cell transplantation (HSCT) and solid-organ transplant (SOT) recipients represent the three major groups of patients at risk of developing invasive fungal infections. Extrinsic factors (radiation, drugs or surgery, resulting in abrasions in the mucocutaneous defensive surfaces of the body), immunosuppression intensity (dose, duration and time sequence of the immunosuppressive regimen) and environmental exposures (community or nosocomial) may also influence which pathogen can produce fungal infection. The risk of infection depends on the general state of immunosuppression and on the epidemiological exposures [4].

#### 1.1.1. From the microbiome

The composition of the microbiome is a unique feature of each individual which is not static and often changes due to the influence of several factors: nutritional, environmental or iatrogenic (complications caused by a certain medical treatment) [5]. There are several interactions between microorganisms and intestinal mucosa host cells [6]. It is important to note that these interactions are dynamic and interconnected. Under homeostatic conditions, the different variables must be counterbalanced giving rise to a balanced and varied

microbiome that is crucial for maintaining health. However, a possible change in the homeostatic state may promote dysbiosis, which is characterized by a significant reduction in microbiome diversity and may lead to the development of pathologies [5].

Among the most important fungi of the microbiome is the yeast *Candida*. Only 20 of the 200 species comprising the genus *Candida* had been linked to human diseases, however, *Candida* species are the most common fungus in humans causing superficial, skin, mucosal infections and systemic infections [7]. *C. albicans* is an ideal immune target because contains a cell wall constituted of polysaccharides, such as chitin, ß-glucan and manna (mannoproteins) and most immune responses are therefore activated and modulated by the cell wall components [8]. Additionally, epithelial cells (first line of defense) induce the expression of antimicrobial peptides, such as histatins, defensins and cathelicidins, which control the growth of *C. albicans* in the commensal phase and during infection [9].

As *C. albicans* is an essentially harmless colonizer of mucosal tissues, the host immune response must know when it is time to remove the invasive hyphal forms or tolerate commensal yeast cells. A stable microbiome is the key to maintaining this balance, which prevents *C. albicans* to switch from a harmless commensal to a pathogen, although, *C. albicans* often causes infection. The pathogenesis process is based on different steps - mucosal surface colonization, overgrowth of fungus leading, ultimately, to tissue invasion and mucosal infection.

Superficial infections caused by *C. albicans* are quite common, affecting 75% of women in reproductive ages, vulvovaginal candidiasis (CVV) is one of the most common infection caused by *Candida*, essentially in patients with HIV [10]. The number of immunosuppressed patients has increased significantly in recent years, being candidiasis one of the fungal infections most frequently present in immunocompromised patients.

Candidiasis is, in fact, an opportunistic infection that can manifest itself in the oral and vaginal mucosa. It can also manifest systemically, in which 90% of the infections are caused by the following species: *C. albicans* - corresponding to 50% of the total, being the most prevalent species -, *C. glabrata*, *C. parapsilosis*, *C. tropical* and *C. krusei*. The frequency of invasive candidiasis varies according to geography and demography of the population [11-13]. *C. albicans* can cause a blood infection, known as candidemia. Candidemia leads to colonization of internal organs, inducing disseminated candidiasis, and

these clinical conditions are extremely serious and fatal to the patient, particularly the most debilitated one. For this reason, antifungals are administered as prevention after bone marrow transplants or abdominal surgeries in order to avoid this type of infection [14]. Despite the developments in the diagnosis and treatment of candidiasis, infection by *Candida* still causes high mortality rates [15].

Nevertheless, the incidence of *C. albicans* species has been declined while *non-albicans* increased. Over the past 20 years, there has been a modification in the rates of *Candida* species isolated from patients with candidiasis, the main cause of this change is the use of fluconazole [12,16-18]. A number of regional, national and global studies have shown that the incidence of *C. albicans* has declined from 70% to 50% [19]. Recent studies have shown that in North America and in many European countries, infections caused by *C. albicans* have been progressively decreasing; on the other hand, infections caused by *C. glabrata* have increased [20,21]. In European countries, the distribution of *Candida* species differs from one country to another (**Table 1**).

***Table 1:*** *Distribution of Candida species in Europe (Adapted from: Epidemiology and risk factors for invasive candidiasis. Ther Clin Risk Manag. 2014, 90, 95-105).*

| Year | Countries | *Candida albicans* (%) | *Candida tropicalis* (%) | *Candida parapsilosis* (%) | *Candida glabrata* (%) | *Candida krusei* (%) |
|---|---|---|---|---|---|---|
| **2002** | Belgium [34] | 55 | 2.8 | 13 | 22 | 2.3 |
| **2004-2009** | Denmark [32] | 57.1 | 4.8 | 3.7 | 21.1 | 4.1 |
| **2010-2011** | Denmark [33] | 52.1 | 4.1 | 4.2 | 28 | 4.8 |
| **1995-1999** | Finland [35] | 70 | 3 | 5 | 9 | 8 |
| **2004-2007** | Finland [36] | 67 | 2 | 5 | 19 | 3 |
| **2004-2005** | Germany [22] | 58.5 | 7.5 | 8 | 19.1 | 1.4 |
| **2006-2011** | Germany ICU [23] | 66 | - | - | - | - |
| **2007-2008** | Italy ICU [29] | 40.2 | 9.8 | 36.9 | 9.8 | - |
| **2009** | Italy [28] | 50.4 | 8.2 | 14.8 | 20.3 | - |
| **1991-2003** | Norway [37] | 69.8 | 6.7 | 5.8 | 13.2 | 1-6 |
| **2008-2009** | Spain [26] | 49.0 | 10.7 | 20.7 | 13.6 | 2.1 |
| **2009-2010** | Spain [27] | 44.7 | 8.2 | 26.6 | 11.5 | 2.0 |
| **2005-2006** | Sweden [24] | 60.8 | 2.0 | 8.9 | 20.1 | 1.2 |
| **1991-2000** | Switzerland [25] | 68 | 9 | 1 | 15 | 2 |
| **1997-1999** | UK and Wales [30] | 64.7 | 4.4 | 7.4 | 16.2 | 2.9 |
| **2008** | UK [31] | 53.7 | 3.2 | 10.7 | 25.8 | 1.0 |
| **2008-2009** | Turkey [38] | 45.8 | 24.1 | 14.5 | 4.8 | - |

### 1.1.2. From the environment

Fungi of environmental origin are globally ubiquitous and their exposure to humans is almost universal. All of these species must be able to adapt to drastic changes in the environment. Host airway exposure to environmental fungus is constant, but very few environmental fungi are capable of causing host disease. *Aspergilllus* spp., *Mucor* spp. and *Cryptococcus* spp. are the fungal species with environmental origin that most frequently cause human infections [40,41].

*Aspergillus* species are globally distributed, and their flexible characteristics facilitate their survival in various environments. These characteristics include: heat and cold tolerance [42,43], drought tolerance [44], tolerance to other stressors [45] and the ability to use a large variety of carbon and nitrogen sources. The ubiquitous and global distribution of *Aspergillus* spp. in external environments makes their exposure practically unavoidable. Although exposure occurs either indoors or outdoors [46], there are crucial risk factors, for example, activities such as demolition or construction of buildings near hospital sites increase the risk of aspergillosis developing in vulnerable patients, once soil disturbance can lead to increased levels of airborne conidia [47].

In invasive pulmonary aspergillosis (IPA), the fungus enters the lungs, invades the lung tissue and damages it, and thus enters the bloodstream and the infection can spread to distal sites, including the skin and, in more severe cases, the brain [48]. Furthermore, chronic pulmonary aspergillosis causes significant damage to lung tissue but, as expected, progresses more slowly than IPA.

The most common species of *Aspergillus* that cause invasive human diseases are *A. fumigatus*, *A. flavus*, *A. niger* and *A. terreus*, but *A. fumigatus* accounts for approximately 90% of all cases of high-risk invasive infections [49]. Invasive aspergillosis has a higher prevalence among bone marrow transplant recipients, presenting high rates of mortality and morbidity [50].

*Cryptococcus* was first identified in 1894 from a chronic granuloma of a tibial bone, since this discovery more than 30 species of *Crytococcus* have been identified; however, only two restricted species have been associated with human diseases: *C. neoformans* and *C. gattii*. *C. neoformans* and *C. gattii* are basidiomycete yeasts and etiologic agents of

cryptococcosis, however, the main etiological agent of this infection is *C. neoformans* which is globally distributed and its exposure is almost universal [51,52]. Cryptococcosis is an invasive mycosis that has a higher incidence in immunocompromised individuals although, in some cases, affects healthy individuals [51].

*C. neoformans* is found in decaying tree cavities and feces of birds, essentially pigeons. These birds can be asymptomatic and yet carry the fungus that consequently cause urban infections. *C. gatti* is not as widely distributed as *C. neoformans*, despite this, is more prevalent in tropical and semitropical regions and often isolated from eucalyptus and koala [51-53].

*Cryptococcus* infections were rare until the appearance of HIV, with less than 300 cases worldwide [54]. However, with the appearance of HIV and consequently the increase of immunocompromised individuals, *Cryptococcus* spp. became a big and serious threat. In the presence of an immune suppression, a latent infection may spread from the lungs to other tissues such as the prostate, skin and eyes. Additionally, this fungus has a predilection for the CNS (Central Nervous System) and may lead to meningoencephalitis. Infections caused by *C. neoformans* and *C. gatti* share a similar pathogenesis [55].

*Mucor* spp. are the fungus responsible for infections commonly known as zygomycosis or mucormycosis. *Mucor* spp. are filamentous fungus found in plants, soils and decaying fruits, *M. amphibiorum*, *M. circinelloides*, *M. hiemalis*, *M. indicus*, *M. racemosus* and *M. ramosissimus* are the most common species [56].

Zygomycosis or mucormycosis includes renal infections, gastritis, pulmonary and mucocutaneous infections. Clinical complications arise when vascular invasion occurs and consequently necrosis of the infected tissue [56]. Diabetes mellitus is a major risk factor for the development of mucormycosis, more specifically 36% to 88% of all patients with mucormycosis are diabetic. Additionally, immunosuppressed individuals are at higher risk of developing this infection with mortality rates of 68% to 100% [57].

### 1.2. Endemic fungi

Endemic fungi are common in specific geographic areas of the world. *Coccidoides* spp. is more common in semi-desert areas, *Histoplasma spp*. is present in tropical regions and *Bastomyces* spp. belongs to temperate climates. Mycoses caused by these endemic fungi (coccidioidomycosis and blastomycosis) are rare in Europe with the exception of travelers, aid workers, archaeologists and immigrants returning from infected endemic areas [58].

In the environment, the appropriate ecological niches for the development of *Blastomyces* spp. is not well defined, however, appear to be similar to *Histoplasma* spp. For example, *B. dermatitidis* and *B. glichristii* reside particularly in freshwater drainage basins and can be isolated in forested areas near lakes and rivers that contain decaying vegetation and acidic soils [59,60]. Additionally, disturbance of contaminated soil leads to spore release and consequently increases the probability of exposure. *Blastomyces* spp., usually, does not use bats or other animals as vectors, but symptomatic infection is frequent in dogs and wild canids.

In endemic regions, the annual incidence of *Blastomyces* spp. ranges from <1 per 100,000 human cases to >100 per 100,000 human cases during sporadic outbreaks [61]. At least half of the reported infections are asymptomatic and surprisingly more frequent in immunocompetent patients. Most of the clinical cases (50 to 75%) of blastomycosis are more restricted to the lungs [62]. Pulmonary blastomycosis typically appears as acute or chronic pneumonia. Despite this, severe clinical cases are more evident in immunocompromised patients.

*Histoplasma* species are often found in basins and have a preference for soils rich in nitrogen, which is naturally supplied by bat or bird feces. Bats probably serve as reservoirs for *Histoplasma* spp. because they are susceptible to pulmonary and disseminated infections caused by *Histoplasma* spp. and, consequently, can release the fungus in their feces [63].

*Histoplasma* spp. represent the etiological agents of histoplasmosis. Histoplasmosis infects not only immunocompromised individuals but also healthy individuals in highly endemic areas. However, there are two specific species- *H. capsulatum* var *capsulatum* and *H. capsulatum* var *duboisii*, which are responsible for most human infections [64].

Hypersensitivity skin tests performed in endemic regions suggest that ~ 90% of their residents have been exposed to *Histoplasma* spp. at least once. Nevertheless, only 1% of exposure cases will result in serious diseases, particularly, in immunocompromised patients [65]. The emergence of HIV increased the incidence of histoplasmosis which proved that *Histoplasma* spp. had a broader geographical presentation than might be expected.

*Coccidioides* species are endemic in arid deserts of the United States and northern Mexico, can tolerate a wide range of soil temperatures and pH, however, have a preference for high salinity and alkaline sandy soils [66]. *Coccidioides* spp. are commonly isolated from animal burrows and carcasses, they can resume the growth of hyphae in the soil after the death of infected animals [67]. Coccidiodomycosis, commonly known as valley fever, is caused by two species- *Coccidioides immitis* and *Coccidioides posadasii*. Hypersensitivity skin tests suggest that ~ 50% of the habitants of endemic areas were probably infected and it is thought that 30 to 50% of those infected habitants are developed symptomatic fungal pneumonia [66,68]. Most cases of coccidioidomycosis are self-limiting pneumonias where dissemination occurs in less than 5% of immunocompetent patients [69]. *Coccidiodes* spp. essentially spread to the CNS, skin, bones and joints [70].

### 1.3. Emerging fungi

The increase in HSCT or SOT and the continued use of immune modifiers, such as tissue necrosis factor (TNF) antagonists for the treatment of chronic inflammatory diseases, has created a new patient population with higher risk of fungal infections [19,71].

*Fusarium* spp. are not often diagnosed in clinical practice as *Candida* spp., *Aspergillus* spp. and *Mucor* spp., but the emergence of this new population seems to be the perfect niche for this emerging species. Infections caused by *Fusarium* spp. are relatively rare but their incidence are increasing mainly immunocompromised populations [19,71].

*Fusarium* is a fungus from the soil and uses the respiratory system as the gateway. This fungus has also been connected with venous access devices, being its prevalence clearly higher in patients who carry a central venous catheter because of its capacity to form biofilms [72]. In immunocompetent hosts, the most common route of infection is the traumatic inoculation in the presence of organic material. On the other hand, airborne conidia, both inside and outside the hospital environment, are the most common source of infection in

immunocompromised patients. *Fusarium* conidia can become aerosolized through water systems such as sinks, showers and drains [73,74].

Clinical manifestations of infections caused by *Fusarium* spp. are not very different from other common fungal infections, a recent review of the characteristics and symptoms of fusariosis showed that spread to the skin was present in 70% of patients. In tissue, *Fusarium* spp. presents itself as a septum hyaline mold (**Figure 1**) similar to *Aspergillus* spp. [75]. Posaconazole and amphotericin B are usually used for the treatment of infections caused by *Fusarium* spp. although some isolates may respond positively to voriconazole [76].

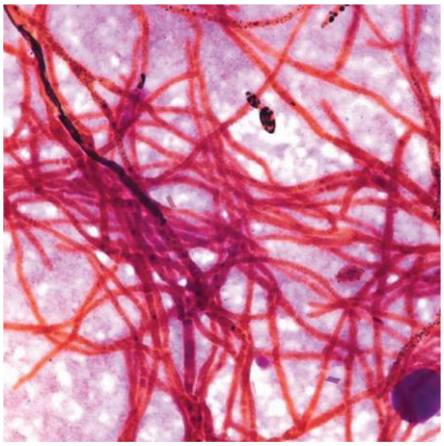

***Figure 1****: Disseminated fusariosis culture showing abundant hyphae (Adapted from: Lockhart et al. Emerging and reemerging fungal infections. Semin. Diagn Pathol.2019, 36, 177-181).*

*Trichosporon* spp. are fungi that usually arise in the form of yeast and are responsible for superficial infections, such as whitepiedra, allergic pneumonia and sometimes invasive infections [77]. Invasive infections of *Trichosporon* spp. were considered the most frequent infections in patients with hematological diseases [78]. Triazole-derived antifungals such as fluconazole have decreased the incidence of these infections [79,80].

The diagnosis of infections caused by *Trichosporon* spp. is difficult mainly due to the absence of specific and characteristic biomarkers of these infections. From the identified 50 species of *Trichosporon*, 17 are clinically relevant [81]. Treating infections caused by *Trichosporon* spp. is difficult as well as challenging because *Trichosporon* spp. appears to exhibit intrinsic resistance to echinocandins and reduced susceptibility to polyenes [82]. *Trichosporon* spp. can also occur in immunosuppression contexts, in newborns and in

various debilitating conditions. *Trichosporon asahii* is associated with a poor prognosis due to its reduced sensitivity to treatment with some azoles.

*Scedosporium*, an emerging pathogen, is considered a potential etiological agent of severe infections in immunocompromised patients and occasionally also in immunocompetent patients. There are two medically important species - *S. apiospermum* and *S. prolificans*, both present in sewage, soil and polluted waters [83].

Scedospirose includes a large group of diseases caused by *Scedosporium* spp., including septic arthritis; pneumonia; endocarditis; peritonitis; meningoencephalitis; meningitis; brain and thyroid abscess and sinusitis. These fungi can colonize patients with pulmonary tuberculosis and cystic fibrosis who already have damaged bronchopulmonary trees. Infections caused by *Scedosporium* spp. can be localized and spread to surrounding tissues or even spread, through the bloodstream, to distant organs [84]. Skin and soft tissue infections caused by *Scedosporium* spp may extend to the tendons, ligaments and bones; the dissemination of these infections is mainly seen in immunocompromised patients; however, cases of disseminated disease have been reported in immunocompetent patients. Treatment of infections by *Scedosporium* spp. is difficult due to the resistance of these fungi to a wide range of antifungal agents [85].

### *2. Candida albicans* virulence and control

As mentioned earlier, *C. albicans* is a commensal fungus that can be isolated from the gastrointestinal tract, oral and vaginal mucosa of healthy individuals, where it resides in balance with the bacterial flora and host immune system. This fungus presents high capacity to survive and proliferate in adverse environments with drastic changes in oxygen, carbon dioxide, pH, osmolarity, nutrients and temperature [86]. *C. albicans* shows different morphologies, such as yeast, pseudohyphae or hyphae (**Figure 2**). The pseudohyphae and hyphae are morphologically distinct; the pseudohyphae contain constrictions at the sites of septation and are considerably larger than the hyphae. On the other hand, hyphae produce long filaments with parallel and unconstrained sides at the site of septation [87].

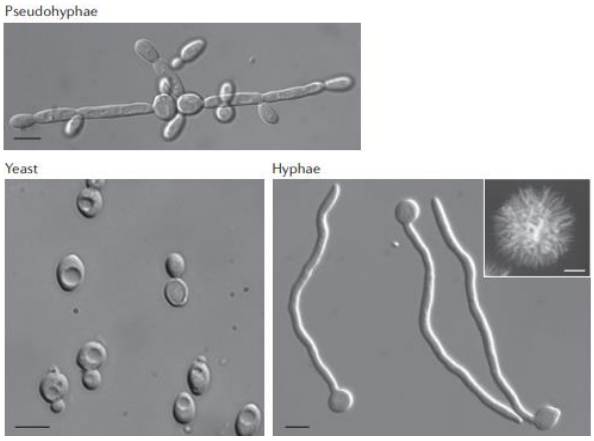


***Figure 2**: Morphology of yeast, hyphae, and pseudohyphae. Scale bars in the main panels represent 5µm, and in the inset on the hyphae, panel represents 1 mm (Adapted from: Sudbery et al. Growth of Candida albicans hyphae. Nat Rev Microbiol. 2011, 9, 737-748).*

The growth of the hyphae comprises two different stages which can be distinguished experimentally. The first stage involves the production of germ tubes and hyphae in liquid medium such as serum at 37 °C. The first stage represents the ability to establish and maintain hyphae growth in the short term. On the other hand, the second stage represents the ability to maintain long-term growth, evaluating the morphology of the colonies after 5 days of growth in serum at 37 ºC [87].

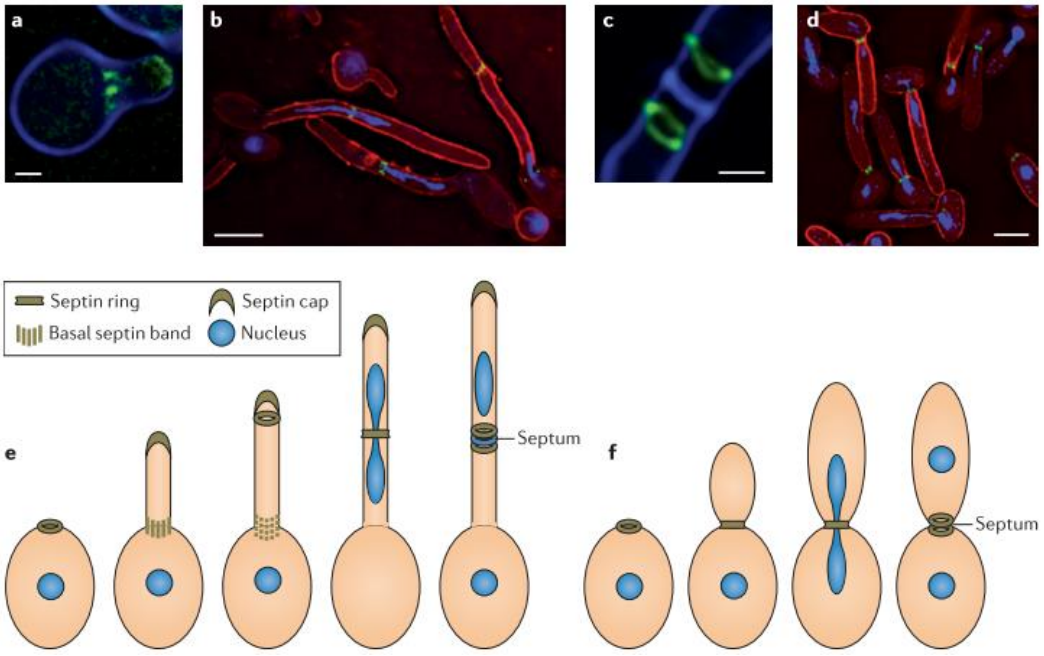


***Figure 3**: Hyphal, **(a)** / **(b)** / **(c)** and **(e)**, and pseudohyphal development, **(d)** and **(f)** (Adapted from: Sudbery et al. Growth of Candida albicans hyphae. Nat Rev Microbiol. 2011, 9, 737-748).*

When the hyphae formation process begins, a single germ tube evaginates from the parent cell (**Figure 3**). The point at which evagination was initiated is marked by a patch of septins that give rise to a band at the base of the germ tube and a cap at the elongated tip (**Figure 3a, e**). The germ tube exhibits highly polarized growth [88]. The evagination occurs before the beginning of the cell cycle, so the cell cycle is started when the germ tube is already formed, at this stage the germ tube can have up to 15-20 μm [89,90]. Parallel to the beginning of the cell cycle, the cap of septins at the tip of the germ tube forms a ring that remains stationary (**Figure 3b, e**). Next, the nucleus of the mother cell migrates to the germ tube, where the first cell division occurs (**Figure 3b, e**); after mitosis, the nucleus of one of the daughter cells migrates back to the mother cell, while the other one migrates to the apical position of the septin ring [90-92] (**Figure 3e**). After this, the spetin ring divides itself in two, and between them a new ring is formed, an actomyiosin ring, composed of actin and myiosin I. This actomyiosin ring contracts and leads to the formation of the primary septum between the septin rings. Following the formation of the primary septum occurs the formation of secondary septum, however, the primary septum is not hydrolyzed, (contrary to what happens on yeast cells) and so the two daughters compartments stay attached to each other (**Figure 3c**). The global organization of growth and development in *C. albicans* hyphae is different from yeast and pseudohyphae [92-94] (**Figure 3d, f**). In addition, both yeast and pseudohyphae grow in synchrony with the cell cycle, while in the hyphae the initial formation of the germ tube occurs before the G1 / S transition [93]. Hyphal growth is stimulated by environmental conditions such as growth at 37 °C, neutral pH, a high concentration of $CO_2$ [7,88,95] and presence of *N*-acetylglucosamine [7,96].

The cell wall of *C. albicans* contains four classes of macromolecules, β(1,6)-glucan, β(1,3)-glucan and small amounts of chitin and mannoproteins. The cell wall of the hyphae has a slightly higher chitin content than the yeast form [96]. Another important feature of *C. albicans* is its ability to form biofilms. Biofilms are a problem in medical practice because they can be formed in artificial heart valves and dentures, presenting resistance to various antifungal agents currently used in clinical practice, including amphotericin B and fluconazole, showing multiple mechanisms of resistance [97].

### 2.1. Virulence factors

An extensive variety of virulence factors allows *C. albicans* to successfully infect several host niches (**Figure 4**). These virulence factors include morphological changes, expression of adhesions and invasins on the cell surface, thigmotropism, formation of biofilms, phenotypic exchange and secretion of hydrolytic enzymes. In addition, a rapid adaptation to fluctuations in environmental pH, metabolic flexibility, powerful nutrient acquisition systems and robust stress response machinery represent important attributes for host infection [98].

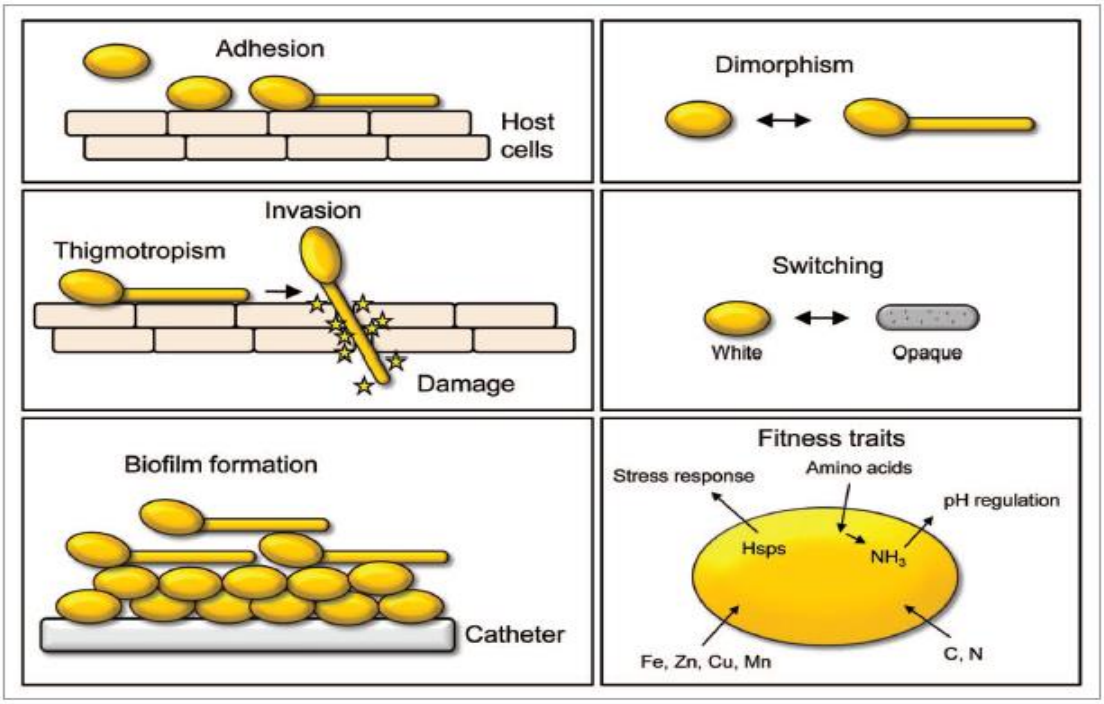


***Figure 4**: Mechanisms of pathogenicity/virulence of C. albicans (Adapted from: Mayer et al. Candida albicans pathogenicity mechanisms. Virulence. 2013, 4, 119-128).*

#### A. Polymorphism

*C. albicans* is a polymorphic fungus that can grow as yeast, pseudohyphae or hyphae. The yeast and hyphae are specially observed during infection, however, have different functions, the role of pseudohyphae in infection is quite undefined [99,100].

It is interesting to note that different environmental signs affect the morphology of *C. albicans*. For example, at low pH (<6), *C. albicans* cells often grow in yeast form, however, at high pH (> 7), the hyphae growth is induced. Other conditions including the

presence of serum or *N*-acetylglucosamine, physiological temperature and $CO_2$ stimulate the formation of hyphae [87]. Both forms are important for pathogenicity, but the hyphae form is more invasive than the yeast form [100].

### B. Adhesins and invasins

*C. albicans* have a specific group of proteins, the adhesins, these proteins mediate the adhesion of *C. albicans* to host cells, to other microorganisms, or to abiotic surfaces. The most common adhesins in *C. albicans* are the Agglutinin-like sequence (Als) proteins. These adhesins form a family consisting of nine members [101,102]. Among the nine Als proteins, Als3 is an adhesin associated with the hyphae form and is especially important for adhesion, Als3 gene expression is upregulated during oral epithelial cell infection *in vitro* and during vaginal infection *in vivo* [103,104]. Hwp1 is another important adhesin of *C. albicans* and serves as a substrate for mammalian transglutaminases.

*C. albicans* is an extraordinary pathogen because it is able to present two different mechanisms to invade the host: induced endocytosis and active penetration [104]. Initially, adhesins such as Als3 bind to their cellular targets (*e.g.* E-cadherin). This initial step ensures that the cells of the fungus are firmly attached to host cell surface, in the second stage *C. albicans* invades the host. In the mechanism of induced endocytosis, the fungus expresses invasins such as Als3 (which also functions as an adhesin) and Ssa1 (expressed on the cell surface, which belongs to the heat shock protein family 70 (Hsp70)) that interact with target receptors, E-cadherin in epithelial cells and N-cadherin in endothelial cells, leading to activation of these receptors. This, in turn, stimulates clathrin recruitment and cytoskeletal reorganization to form an invasion pocket in which *C. albicans* invades the host cell [105, 106]. The factors mediating the active penetration in host cells are not known yet, however, active penetration is known to be a fungal process that specifically requires viable *C. albicans* hyphae [106].

In conclusion, the invasion of host cells by *C. albicans* depends on two important and complementary mechanisms: induced endocytosis measured essentially by Als3 and Ssa1 and active penetration mediated by mechanisms not known yet.

### C. Biofilm formation

*C. albicans* is able to form biofilms on abiotic or biotic surfaces. Catheters, dentures (abiotic) and mucosal (biotic) cell surfaces are the most affected substrates [107]. The formation of biofilms is sequential and involves the adhesion of the yeast to the substrate and its proliferation, formation of hyphae (in the upper part of the biofilm), accumulation of extracellular matrix material and finally the dispersion of yeast cells from the biofilm complex. It is important to note that mature biofilms are much more resistant to antimicrobial agents and immunological factors of the host compared to planktonic cells, consequently, they are more difficult to treat [108].

### D. Secreted hydrolases

After adhesion of the yeast to the surface of the host cells and hyphal growth, *C. albicans* hyphae can secrete hydrolases that, by themselves, facilitate the active penetration in the host cells. Additionally, the secreted hydrolases may possibly increase the efficiency in the acquisition of extracellular nutrients allowing the survival of the microorganism. Proteases, phospholipases and lipases are three classes of hydrolases secreted by *C. albicans* [109-111].

Secreted Aspartic Proteins (Saps) are a family of proteins composed of ten members, Sap1-10. Sap1-8 are released into the surrounding environment while Sap9 and Sap10 remain attached to the cell surface [111]. The contribution of Saps to the pathogenicity of *C. albicans* generates some controversy, it has recently been shown that Saps are not required for invasion of the reconstituted human epithelium (RHE) and that Sap1-6 are dispensable for virulence in disseminated candidiasis in animal models [112].

There are four distinct families of phospholipases (A, B, C, and D), but only five members of class B (PLB1-5) are extracellular and may have an important role in the pathogenicity because they facilitate disruption of host membranes [113].

*C. albicans* also produces lipases, these constitute the third family of hydrolases secreted by the fungus. This family consists of 10 members (LIP1-10) [114].

### E. pH sensing

In human hosts, *C. albicans* is exposed to an endogenous pH ranging from alkaline to acid. However, the fungus is able to adapt to changes in pH. It is known that the pH of human blood and tissues is slightly alkaline on the other hand, the pH of the digestive tract varies from extremely acidic (pH 2) to more alkaline (pH 8) and the pH of the vagina is acid [115]. The change from neutral to alkaline pH can cause severe stress in *C. albicans* causing malfunction of pH-sensitive proteins and impair nutrient acquisition. There are two β-glycosidases (Phr1 and Phr2) of the cell wall of the fungus that are extremely important for adaptation of the fungus to changes of pH [116]. At a neutral-alkaline pH, the Phr1 is expressed, on the other hand, at an acid pH the Phr2 is expressed. In the case of systemic infections, Phr1 is required while Phr2 is essential for vaginal infections [117].

*C. albicans* is a multifaceted and complex fungus because it is not only able to feel and adapt to the environmental pH, it can also modulate the extracellular pH actively alkalizing its surrounding environment and thus, self-induce the formation of hyphae [118].

### F. Metabolic adaptation

The survival and growth of any microorganism depend on the acquisition of nutrients. Metabolic flexibility is a fundamental precondition for pathogenic fungi during the infection of several host niches. Glycolysis, gluconeogenesis and other starvation responses are all thought to contribute to host colonization, but their contribution may be highly specific and still not well described in the literature [119-121].

*C. albicans* is a commensal microorganism and is predominantly found in the gastrointestinal microbiome of healthy individuals. The gastrointestinal tract has a high concentration of nutrients, but the growth of the fungus is controlled through competition with other microorganisms of the intestinal flora. In disseminated candidiasis, in individuals with the compromised immune system, *C. albicans* gains direct access to the bloodstream, blood is rich in glucose (6 to 8 mM) which by the way is the "preferred" nutrient of most fungi [119]. In addition to its capacity to metabolic adaptation, the fungus also has several ways of protecting itself from macrophages, inhibiting the production of antimicrobial effectors and inducing the formation of hyphae. The hyphae formed inside the phagocytic cells can cross the host immune cells by mechanical forces and thereby escape [122,123]. It

is important to note that in the case of systemic candidiasis, fungal cells can spread to all organs of the human host and each organ has a potentially different nutrient availability. For example, in the liver, *C. albicans* has access to large amounts of glycogen, the main glucose storage molecule [120-122]. However, in other tissues, *C. albicans* faces relatively low glucose concentrations and uses other alternative metabolic pathways to obtain other nutrients from the host such as proteins, amino acids, lipids, and phospholipids [123].

### G. Environmental stress response

The response to environmental stress plays a key role in the survival and virulence of *C. albicans*, promoting the adaptation of the fungus to changing conditions, protecting it against stress factors induced by host cells. Phagocytic cells of the immune system (*eg*, macrophages) induce oxidative and nitrosative stress. Stress-responsive regulatory pathways have been shown to be essential for the adaptation of stress induced by host cells and for the virulence of the fungus [124].

In response to osmotic stress, *C. albicans* accumulates glycerol intracellularly to compensate for water loss as a consequence of the chemical gradient. Glycerol biosynthesis is mediated by the enzymes glycerol 3-phosphatase (Gpp1) and glycerol 3-phosphate dehydrogenase (Gpd2) [105].

It is known that some reactive oxygen species (ROS), like superoxide, hydroxyl radicals and peroxide cause oxidative stress in the yeast. To respond to oxidative stress caused by these species, *C. albicans* induces the synthesis of superoxide dismutases (Sod1 and Sod5) and catalase (Cta1) to combat the toxic effect of these ROS [125-127]. On the other hand, neutrophils also produce reactive nitrogen species (RNS) which in turn induce a response to nitrosative stress in phagocytized *C. albicans* cells [127].

## 2.2. Therapeutic strategies to control *C. albicans* infections and antimicrobial resistance in fungi

Currently, systemic fungal infections represent a challenging problem in clinical practice [128-130]. Additionally, in the last decade, resistant fungi emerged, such as azole-resistant *Candida* spp. Antifungal resistance is a broad concept that describes the failure of fungal infection to respond to antifungal therapy. Clinical resistance of fungi is typically

observed in patients with persistent and profound immune defects or infected prosthetic material [130].

The broader family of antifungal agents are the azole derivatives, such antifungals are capable of breaking the cell membrane, inhibiting the enzyme lanosterol 14-α-demethylase, which is responsible for the synthesis of ergosterol (**Figure 5**) [131]. The ergosterol is one of the major constituents of the fungal cell membrane. Imidazoles (miconazole, clotrimazole, ketoconazole, and econazole), triazoles (fluconazole and itraconazole) and posaconazole are part of the azole derivatives and are approved by the US Food and Drug Administration (FDA) and the European Medicines Agency [132,133]. Fluconazole and itraconazole have been frequently used for the treatment of systemic fungal infections because of their favorable oral bioavailability. A high percentage of fungi have developed resistance to fluconazole [133]. *C. albicans* has demonstrated resistance to azole in patients with HIV or with oropharyngeal candidiasis. Non-*albicans* species such as *C. glabrata* and *C. krusei* have shown reduced susceptibility to fluconazole [134].

The first line drugs for the treatment of invasive candidiasis is the echinocandins. However, resistance to this antifungal agent has been observed in patients with *C. glabrata, C. tropicalis, C. albicans* and *C. krusei* infections. In the case of *C. glabrata*, resistance to echinocandins increased from 4.9% to 12.3% within 9 years (2001-2010) [135]. Echinocandins (caspofungin, micafungin and anidulafungin) represent a group of lipopeptide antifungal agents that are able to inhibit fungal wall synthesis by blocking ß(1,3)glucan synthase. Inhibition of this enzyme affects the structural integrity of fungal wall, which ultimately results in the cell's osmotic lysis [136]. This family of antifungals has been approved by the FDA for the treatment of invasive and esophageal candidiasis [137-139]. On the other hand, the polyenes (nystatin and amphotericin B) bind to ergosterol destroy it and consequently lead to the production of aqueous pores. In this way, the cellular permeability is altered resulting in the inappropriate leakage of cytosolic components and consequently in the death of the microorganism [139].

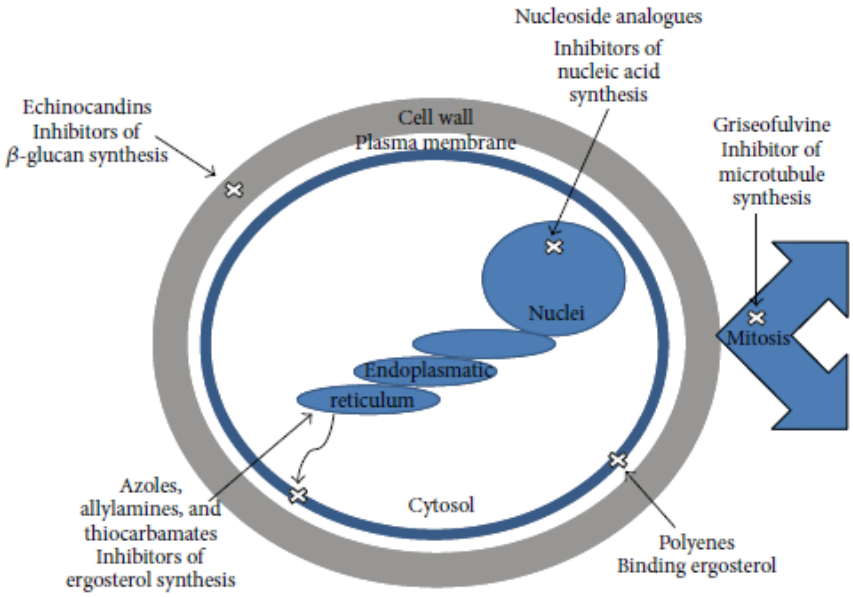


***Figure 5****: Mode of action of several antifungal agents (Adapted from: Spampinato et al. Candida Infections, Causes, Targets, and Resistance Mechanisms: Traditional and Alternative Antifungal Agents. BioMed Research International. 2013, 2013, 204-237).*

Amphotericin B has been used in clinical practice for more than 30 years, but the frequent and inappropriate use of this drug has led to the development of minimal resistance, this resistance depends on the species [140]. However, side effects and toxicity have always been the main difficulties associated with the use of conventional amphotericin B [141]. It has been reported in the literature that *C. glabrata* and *C. krusei* are usually sensitive to this drug, although they have higher MICs values for polyenes than *C. albicans* [140]. To fight this, higher doses of amphotericin B were recommended by the Infectious Diseases Society of America for the treatment of infections caused by *C. krusei* and *C. glabrata* [142].

There are also antifungal agents capable of inhibiting DNA synthesis. Flucytosine is one of this type of antifungal agent. Flucytosine is itself a pyrimidine analogue and is transported into the fungal cells via cytosine permeases. Thereafter, deamination of flucytosine results in the 5-fluorouracil which is then phosphorylated and forms the 5-fluorodeoxyuridine monophosphate. This fluorinated nucleotide inhibits DNA synthesis [143].

Griseofulvin (isolated from *Penicillium griseofulvum*) inhibits the production of microtubules and consequently the formation of the achromatic spindle. Briefly, griseofulvin is able to inhibit fungal mitosis and thus cell growth [144].

If the topical antifungal drugs can be used to successfully treat superficial infections, on the other hand, the oral or intravenous (IV) preparations are commonly used to treat systemic infections. Specific pharmacokinetic parameters of some antifungal agents are used to treat systemic candidiasis (**Table 2**). It is important to note that the routes of administration and excretion are often important in selecting an appropriate antifungal agent [145]. There are some drugs, depending on their solubility of the drug, that are only available as oral preparations (*eg*, flucytosine and posaconazole), only as IV preparations (*eg*, amphotericin B, micafungin, caspofungin and anidulafungin) or may even be given through both routes (*eg*, fluconazole and itraconazole).

***Table 2**: Pharmacokinetic parameters of representative antifungal agents (Adapted from: Spampinato el al. Candida Infections, Causes, Targets, and Resistance Mechanisms: Traditional and Alternative Antifungal Agents. BioMed Research International. 2013, 2013, 204-237).*

| Drug family | Drug | Adm route | Oral bioavailability (%) | $C_{max}$ µg/mL | Protein binding (%) | Half time (h) | Elimination |
|---|---|---|---|---|---|---|---|
| **Azoles** | Fluconazole | Oral | >90 | 0.7 | 10-12 | 27-31 | Urine [147,148] |
| | Itraconazole | Oral | >55 | 1.1 | 99.8 | 21-64 | Hepatic [146] |
| | Voriconazole | Oral | >90 | 4.6 | 60.0 | 6 | Renal [147,148] |
| | Posaconazole | Oral | >98 | 7.8 | 99.0 | 15-35 | Feces [147,148] |
| **Echinocandins** | Caspofungin | IV | <5 | 9.5-12.1 | 96.0 | 10.6 | Urine [146,147] |
| | Micafungin | IV | <5 | 7.1-10.9 | 99.8 | 11-17 | Feces [146-149] |
| | Anidulafungin | IV | <5 | 3.4-7.5 | 84.0 | 18.1-25.6 | Feces [146-149] |
| **Polyenes** | Amphotericin B | IV | <5 | 1.5-2.1 | >95 | 6.8-50 | Feces [147,150] |
| **Nucleoside analogues** | Flucytosine | Oral | 76-78 | 80 | 4 | 3-6 | Renal [143] |

In case of infections caused by *Candida* species, the potential pharmacological strategies include the use of novel antifungal formulations (for example, amphotericin B lipid complex) and combined therapies of one or more antifungal agents such as fluconazole + flucytosine, amphotericin B + flucytosine, caspofungin + liposomal amphotericin B, caspofungin + fluconazole and amphotericin B + fluconazole [150].

## 3. Photodynamic Therapy (PDT)

Photodynamic therapy (PDT) it is a therapeutic approach based on the systemic or topical administration of a non-toxic dye known as photosensitizer (PS) to a patient and its action occurs when the tissue or surface area to treat are illuminated with light with adequate wavelength. For the occurrence of the photodynamic action it is also needed to be present molecular oxygen. In fact, the combination of these three components (dye, light and oxygen) leads to the generation of cytotoxic reactive oxygen species (ROS) that are capable of causing cell death and consequently tissue destruction. The use of PDT for the treatment of cancer is particularly attractive because of its selectivity, since the PS in general is concentrated within the malignant tissue [151].

Antimicrobial photodynamic therapy (aPDT) can be also an alternative approach to the conventional antimicrobial methodologies. In fact, the increased resistance to antimicrobial agents due to inadequate prescribing or overuse of these agents has become a public health problem. In addition, the frequent transmission of microorganisms due to global travel increases the need for the development of new methods that are less expensive and non-resistant. Thus, aPDT appears to be a promising alternative approach to inactivating microorganisms, which has been shown to be effective in the photoinactivation of bacteria, viruses, fungi, and protozoa [152]. Antimicrobial photodynamic therapy is based on the same principle as photodynamic therapy (PDT) of tumors to generate reactive oxygen species (ROS), namely singlet oxygen ($^1O_2$) to photoinactivate the microorganisms. The fact that aPDT is a multi-target approach gives it a powerful advantage over conventional antimicrobial approach, in other words, this therapy acts on a variety of biochemical targets (extra and intracellular structures) and therefore prevents the development of resistance mechanisms [153,154]. The main targets differ according to the microorganism to be treated. In the case of bacteria and viruses the main targets are extracellular structures such as cell walls, cell membranes, protein capsids and lipid envelopes. These extracellular structures will suffer irreversible damages causing the leakage of the intracellular content or the inactivation of membrane transport systems and enzymes [155]. Nucleic acids can be also a target of aPDT, even for bacteria that are capable by the action of an efficient DNA repairing systems to repair some damages produced in their nucleic acid chain [156-158].

### 3.1. Mechanisms of action of aPDT

As mentioned above, the photodynamic approach is based on the combination of three components, a non-toxic molecule, called photosensitizer (PS), light with a wavelength suitable for excitation of the PS (usually from visible to infrared) and molecular dioxygen ($^3O_2$) (**Figure 6**). Initially the PS absorbs a photon (A) and changes its energetic stated, from a fundamental state ($S_0$) to an excited singlet state ($S_n$). Then the PS can return to the ground state ($S_0$) by emitting light (fluorescence emission) or by heat dissipation due to internal conversion. Otherwise, the excited PS ($S_1$) can be converted through an intersystem crossing (ISC) process to the excited triplet state ($^*T_1$). From this state ($^*T_1$), the PS can return to its ground state ($S_0$) by light emission (phosphorescence emission), by electrons transfer with the surrounding substrates, producing radical species (type 1 mechanism) or by energy transfer with the molecular dioxygen ($^3O_2$) producing singlet oxygen ($^1O_2$) (type 2 mechanism) [161].

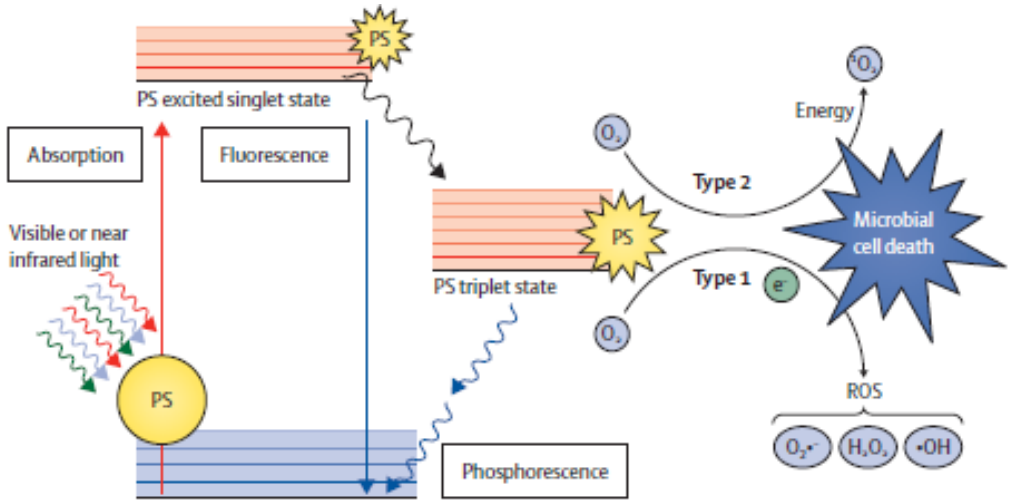


***Figure 6**: Mechanism of photodynamic action (Adapted from: Bonnett et al. Chemical Aspects of Photodynamic Therapy, Gordon and Breach Science, Amsterdam. 2000, 324).*

In the type I mechanism, the charge (*eg*. electrons) is transferred to neighboring substrates leading to the formation of superoxide radicals ($O_2^{\bullet-}$) which can give rise to hydrogen peroxide ($H_2O_2$), the precursor of free hydroxyl radicals ($^\bullet OH$). In the type II mechanism, the energy is directly transferred to the molecular dioxygen ($^3O_2$) leading to the formation of the singlet oxygen ($^1O_2$) which represents the energized molecular oxygen (+ 0.98 eV = + $1.57x10^{-19}$ J). Once the PS is again on its ground state ($S_0$) can absorb a new photon and start a new photocatalytic cycle [162] (**Figure 6**).

### 3.2. Photosensitizers

As mentioned above, the photosensitizer is a dye capable of interacting with light and can be of natural or synthetic origin. In general, all PS must have good light absorptions and should be able to produce adequate amounts of $^1O_2$ for the biological action. The photosensitizing properties of several organic dyes such as Rose Bengal (RB), eosin, methylene blue (MB), fullerenes, porphyrin derivatives (natural or synthetic origin) and phthalocyanines (ZnPc) have been evaluated. The efficiency of photodynamic treatment in microorganisms depends also on the structural characteristics of the PS, such as the presence or absence of positive charge and its distribution in the periphery of the macrocycle. The peripheral positions of the macrocycle may greatly affect the kinetics and extent of binding to the microbial cells [163]. **Figure 7** shows several photosensitizers and their chemical structures.

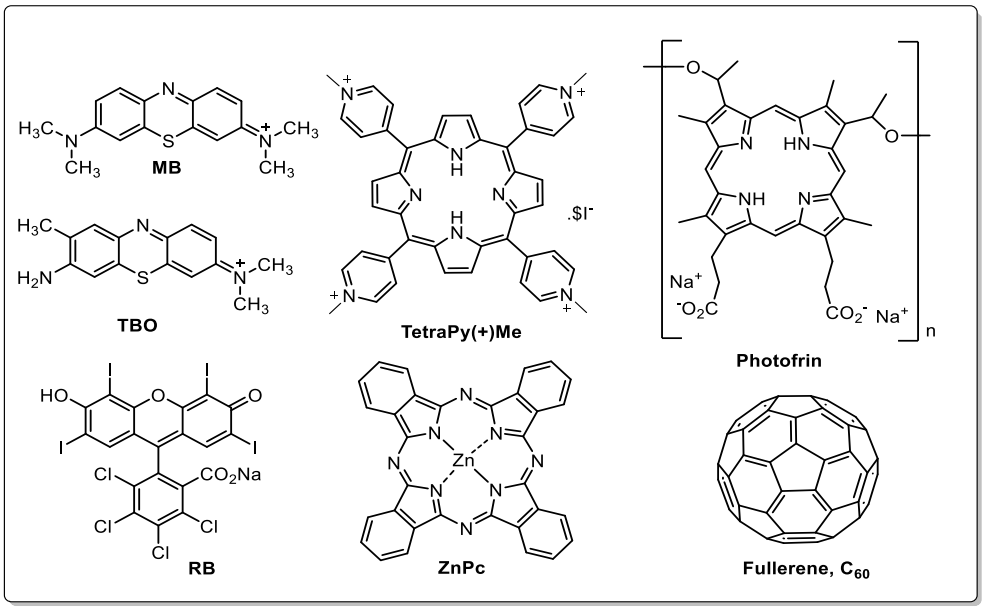


***Figure 7**: Chemical structure of several photosensitizers study for the photoinactivation of microorganisms.*

### A. Phenothiazinium derivatives

The first phenothiazine dye (methylene blue) was developed by Heinrich Caro in 1870 [164]. MB show strong absorption in the red region of the electromagnetic spectrum (600-680 nm), which allow the greater light tissue penetration and good singlet oxygen generation. Methylene blue (MB) or toluidine blue (TBO) are examples of phenothiazinium derivatives used in PDT [165].

### B. Tetrapyrrolic derivatives and analogues

Porphyrins are among the most promising tetrapyrrolic compounds used in photodynamic therapy [164-167]. Porphyrins are a class of aromatic heterocyclic photosensitizers with four pyrrolic type subunits linked by four methinic bridges [167], and participate in vital biochemical processes. Additionally, these compounds are finding applications in different fields ranging from artificial photosynthesis, catalysis, sensors, to photodynamic therapy applied to the treatment of cancer or inactivation of microorganisms. The porphyrin derivatives used as photosensitizers can be divided into two groups: natural porphyrins (*eg*.Photofrin) and synthetic porphyrins (e.g. neutral, cationic or anionic *meso*-tetraarylporphyrins) [152]. The porphyrin "backbone" is essentially hydrophobic, and to overcome the problems associated with this feature, structural modifications of the porphyrinic nucleus such as the addition of peripheral sub-constituents are employed (such as carbohydrate residues or charged groups) used to control the solubility in water and its affinity for the target cells. There are several examples of chemical modifications of porphyrins such as TetraPy(+)Me with four positive charges (**Figure 7**) [168]

Chlorins are structurally identical to porphyrins, but with one of the pyrrolic unit reduced [167]. This group of PSs exhibits a peak absorption at approximately 405 nm (Soret band) and lower absorption peaks at wavelengths greater than 650 nm [169,170]. The cationic derivatives of chlorin-$e_6$ are frequently used in aPDT as is the case of Photodithazine® [171].

Phthalocyanines are heterocyclic compounds and hydrophobic so they must be combined with a solubilization methods to be used as PS [172]. A good example of a phthalocyanine-based PS is the zinc(II) phtalocyanine (ZnPc) **Figure 7**.

**C. Xanthene derivatives**

Xanthene derivatives usually exhibit intense absorption in the green spectrum and act essentially according to type II mechanism such as erythrosine, eosin Y, rose bengal (RB), but their negative charge difficult their use as PS (**Figure 7**) [173].

**D. Fullerene derivatives**

Fullerenes are molecules made essentially from carbon atoms, the absorption peak of these compounds is between the UV and visible spectrum. Fullerene $C_{60}$ (**Figure 7**) is one

of the most studied fullerenes and has several applications through the addition of positive charges [174]. Fullerenes act according to the type I and type II mechanisms depending on chemical modifications and solvents [175]**.**

**E. Phenalenone derivatives**

Phenalenone chromophores are natural compounds synthesized by plants so they can defend against pathogens using the sun to form singlet oxygen [176]. Initially, the PNS [1H-Phenalen-1-one-2-sulfonic acid] was used as the standard PS for photophysical measurements and was not usually used for aPDT due to its negative charge. The need to produce phenolenone derivatives positively charged and soluble in water has arisen and SAPYR-phenalenone, a cationic derivative, was synthesized. SAPYR is a PS that acts exclusively by type II mechanism and is water soluble [177].

**3.3. aPDT in *C. albicans* inactivation**

Fungi are complex microorganisms and therefore become more challenging aPDT targets than bacteria. Their inactivation seems to be less dependent on the binding of PS to cells [153,178, 179]. Free PS induces some initial changes in the plasma membrane and thus can penetrate the cell and cause more extensive damage in more complex subcellular structures such as mitochondria or nucleus [180,181]. From this point of view, the photoinactivation of fungi appears to be more complex since the action of PS is preferentially in more complex intracellular structures. In most microorganisms, such as bacteria, the main targets of aPDT are extracellular structure and so the photosensitizer does not need to penetrate the cell since the adhesion of PS to these structures is more than enough for the photoinactivation of the microorganism [152]. Regarding the required photosensitizer concentrations, these are known to be similar or slightly higher than those used against bacteria and viruses [152].

*C. albicans* has been shown to be susceptible to aPDT in their planktonic [182,183] and biofilms forms [183,184]. Antimicrobial photodynamic therapy has been shown to be a promising therapy for preventing biofilm formation by reducing their adhesion capacity [184]. In addition, other *Candida* species appears to be susceptible to aPDT-*C. dubliniensis*, *C. tropicalis* and *C. krusei* [185].

Some salts seem to potentiate the photodynamic effects of this fungus. Recent studies have shown that the addition of KI appears to increase the antimicrobial capacity of this therapy for *C. albicans* planktonic forms and biofilms [182]. The haematoporphyrin [179], Photofrin [186,187], *meso*-tetrakis(*N*-methyl-6-quinolinyl)-substituted porphyrins and chlorins derivatives [187], quaternary ammonium derivatives of *meso*-tetrapyridylporphyrin [188], porphyrin bearing a fluconazole unit [189] and ALA [190] are some of the PS used *in vitro* assays that efficiently inactivate *C. albicans*.

## 4. Blood

Human blood represents an important tissue with vital functions; it is responsible for the transport of cells and molecules between parts of the body. Oxygen, carbon dioxide and glucose are among the most vital molecules transported in this tissue.

### 4.1. Blood compounds

Donated blood can be fractionated into several components such as fresh frozen plasma; platelet concentrates; concentrated red blood cells and cryoprecipitate. Initially donated blood was not processed into these components and was defined as "whole blood". Currently, most EU countries process donated blood into fractions of red blood cells, platelets and plasma [191]. Plasma constitute about 55% of whole blood, the red blood cells about 45%, and white blood cells less than 1% [192]. Plasma is clinically defined as the aqueous fraction of the blood and contains several proteins and factors important to several vital processes [193]. The main role of blood plasma is to take nutrients, hormones and proteins to the different parts of the body [194].

Due to hemorrhagic diseases, anemia, major surgeries, *etc*, many patients are submitted to blood transfusions that can occur by transfusion of the whole blood or more commonly, transfusions of blood components such as erythrocytes, plasma or platelets. In some cases, multiple blood transfusions are required to meet the body's needs. Transfusion of erythrocytes concentrates are used in hypoxia by blood loss after trauma or surgery [195-197]. These concentrates can be stored for 35 days at 2–6 °C in red cell preservation solutions [197-199]. Platelets transfusion are used for preventing or treating bleeding in patients with thrombocytopenia or abnormalities of platelet function [200,201]. It can be concentrated from plasma or by plateletpheresis from single donor and stored at 22 °C for 5 days [201].

Plasma transfusions are required to correct deficiencies of clotting factors in patients with severe bleeding. Plasma can be frozen promptly, stored at -18 °C during 5 years and could be defrosted before use [197].

In several countries of EU the typical process of blood donations consists in a collection of 450-500 mL of donor blood for a pack with 63 mL of an anticoagulant solution such as Citrate Phosphate Dextrose or Citrate Phosphate Adenine. Dextrose is similar to fructose and chemically identical to glucose, which is a blood sugar. Citrate is very important because it is able to bind to calcium while adenine and glucose are responsible for the metabolism of red blood cells during storage. Next, the total blood can be filtered to remove the leucocytes and the largest part of the plasma is collected [194].

### 4.2. Quality control of blood

The clinical use of blood requires a rigorous evaluation based on quality indicators. These indicators (of quality and performance) are very useful for clinical practice, quality indicators can and should be used to monitor and evaluate the quality of the clinical transfusion process and the correct compliance with clinical guidelines. There are two different types of indicators: internal and external indicators [194]. Internal indicators are used to ensure the quality of the clinical transfusion process, they are vital for critical process steps and for the professionals involved. They must be specific, detailed, educational and effective. On the other hand, external indicators provide information for external control agencies such as health inspection and / or hospital comparisons (comparative reference process). These indicators should provide monitoring and information about process quality, measure global aspects and require good validation [194].

In European Union countries, the availability of the blood components depends entirely on voluntary donation. Currently, there is an alarming decrease in the availability of blood, this decrease is mainly due to the aging of the population and the insertion of new prevention measures in relation to the safety of the receiver [195,203].

Blood collected from the donor may contain microorganisms, mainly from intestinal bacteria, pre-symptomatic infections or from exogenous bacteria, in particular from the skin, but also viruses and parasites (**Table 3**) [203]. There are few studies reporting fungal infections transmitted by blood transfusions. In 2011 a statistical study analyzed 86 patients

with severe abdominal sepsis and severe pancreatitis, 23% of these patients were colonized by *Candida* and 8% of the patients developed candidiasis after transfusion of at least four volumes of red blood cells [204]. .

***Table 3**: Pathogens transmitted through a blood transfusion (Adapted from: Fred et al. Pathogens transmitted in red blood cell transfusions: An up-to-date table. Baylor Univ Med Cent Proc. 2018, 31, 307-309).*

| Viruses | Bacteria | Protozoa |
|---|---|---|
| Chikungunya virus | *Anaplasma phagocytophilum* | *Babesia microti* |
| Colorado tick fever virus | *Bartonella* species | *Leishmania donovani* |
| Cytomegalovirus | *Borrelia recurrentis* (relapsing fever) | Malaria |
| Dengue virus | *Brucella* species | *Mansonella perstans* (microfilaria) |
| Epstein-Barr virus | *Clostridium perfringens* | *Toxoplasma gondi* |
| Hepatitis A virus | *Enterobacter cloacae* | *Trypanosoma cruzi* (Chagas) |
| Hepatitis B virus | Enterococci | |
| Hepatitis C virus | *Klebsiella pneumoniae* | |
| Hepatitis D virus | *Propionibacterium acnes* | |
| Hepatitis E virus | *Pseudomonas* species | |
| Hepatitis G virus | *Rickettsia rickettsii* | |
| Human herpesvirus 8 | *Serratia liquefaciens* | |
| Human immunodeficiency virus | *Staphylococcus aureus* | |
| Japanese encephalitis virus | *Treponema pallidum* (syphilis) | |
| Parvovirus | *Yersinia enterocolitica* | |
| SEN virus | | |
| Tick-borne encephalitis virus | | |
| Torque-tenovirus | | |
| West Nile virus | | |
| Yellow fever vaccine virus | | |
| Zika virus | | |

Initially, the risk of transfusion-transmitted infections (TTI) was assumed to be unavoidable, but currently the risk of these infections is lower .Since the early 1960s, blood banks have been struggling with strategies to reduce the risk of transfusion-transmitted infections [205]. Donor assessment, laboratory screening tests and pathogen inactivation procedures are currently done to reduce the risk of TTI. These tools are considered crucial to reducing the risk of TTI, although they do not completely eliminate all the risks. However, donor screening, as well as the inactivation of pathogens of blood components, need to be balanced against unnecessary loss of donors. All these efforts should be supported by national and international networks of haemovigilance to facilitate the identification of

emerging threats of transfusion-transmitted infections, simplifying the monitoring of the transfusion process [206].

The report of Blood and Transfusion Medicine Services Activity in 2017 published by the Portuguese Institute of Blood and Transplantation show that the oral interview to the donor's is not so sensitivity as the analytical tests performed, but the interview represents an important screening mechanism for the elimination of potentially infected donors (**Table 4**) [207].

***Table 4**: Number of donations refused after clinical screening between 2012-2017 (Adapted from: Instituto Português do Sangue e da Transplatação I. Relatório de Atividade Transfusional e Sistema Português de Hemovigilância. 2017).*

| | **2012** | **2013** | **2014** | **2015** | **2016** | **2017** |
|---|---|---|---|---|---|---|
| **Low levels of hemoglobin** | 22408 | 17641 | 16940 | 13643 | 13973 | 13157 |
| **High risk behavior** | 2078 | 1862 | 2323 | 6382 | 4154 | 5900 |
| **Travels** | 2475 | 1429 | 1713 | 1888 | 1962 | 3522 |
| **Flu syndrome** | 4714 | 4203 | 3760 | 3605 | 3669 | 3118 |
| **Self-exclusion** | 234 | 173 | 285 | 89 | 125 | 245 |
| **Others** | 66006 | 53143 | 53111 | 41924 | 39292 | 32762 |
| **Total donations rejected** | **97915** | **78451** | **78132** | **67531** | **63175** | **58704** |

The **Table 4** shows that the total of allogeneic donations rejected after clinical interview was reduced from 2012 until 2017, the highest number of rejected donations occurred in 2012 and the lowest number of rejected donations occurred in 2017. There is also an increase in the number of donations rejected by travel and high-risk behavior between 2012 and 2017. In 2017, 1246 adverse reactions in donors were reported by the Blood Services [207]. Although the process currently implemented in transfusion medicine is safe and effective, it is extremely necessary develop effective methodologies to improve and / or support the disinfection process of whole blood and its components in order to prevent the transmission of infectious diseases during the transfusion process.

## 5. aPDT as an alternative approach to disinfect blood

The usual treatments for inactivate pathogenic microorganisms in blood and blood derivatives were established essentially to inactivate viruses. The World Health Organization (WHO) recommends the screening for the presence of Human Immunodeficiency Virus (HIV), Hepatitis B Virus (HBV), Hepatitis C Virus (HCV) and also the bacterium *Treponema pallidum* , for all blood donations [208]. However, the collected blood may contain other pathogenic agents like fungi [209], parasites [210] and several bacteria [197], [211- 214].

Currently, there are some available methods for the reduction of pathogens in blood products, but they are approved essentially for plasma. The general method combines the use of tri(*n*-butyl)phosphate and detergent Tween 80 and can be only used in plasma or protein concentrates. Due to the bad effects that these chemicals promote in the erythrocyte membranes and platelets, they must be removed after treatment [199,215,216]. The use of ultraviolet (UV) light is also an alternative and this methodology is essentially applied in plasma and platelet disinfection. The irradiation causes damage to the microbial genetic material avoiding its replication; however, this technique produces free radicals that are very cytotoxic [197,199,217].

Another approach for blood disinfection is the aPDT. In the present days, only three PSs, amotosalen (a psolaren), riboflavin (or vitamin B2) and methylene blue (MB), are approved for blood disinfection and can only be used for plasma and platelets treatment. The aPDT with amotosalen and riboflavin requires the use of UV (UVA) and UV (UVB), respectively, which may lead to the formation of harmful free radicals. Despite this, amotosalen and riboflavin were approved in Europe for platelets and plasma disinfection [218-220]. Contrarily, aPDT with MB uses visible light instead of UV light and it is also approved for pathogen inactivation in plasma units [218,221]. In this case most enveloped viruses are inactivated, but non-enveloped viruses, intracellular viruses, protozoa, fungi and bacteria remain unaffected [218,222]. Although it is approved for the disinfection of plasma in several European countries, in France it was removed from the market due to allergic reactions detected in a few patients that received plasma treated with MB [219]. aPDT using cationic porphyrins such as Tri-Py(+)-Me has been shown to be effective in inactivation of gram positive (*S. aureus*) and gram negative (*E. coli*) bacteria in whole blood [197],

Regarding to red blood cells, a recent study showed that the use of S-303, a positively charged synthetic alkylating agent, can disrupt the pathogen genetic material in erythrocytes [219,223]. Besides these promising studies, no well-established method for whole blood disinfection was approved.

Although already exist some studies showing that aPDT appears to be a promising therapy for bacterial inactivation in whole blood, as far as we know there are no studies showing the efficacy of this therapy in inactivation of *C. albicans* in whole blood.

## 6. Aims

In this work we set out to achieve several goals that were essentials to establish important milestones for success of this thesis:

- Evaluation of photodynamic effect of 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin tri-iodide (Tri-Py(+)-Me) and a PS formulation (FORM) in the photoinactivation of *C. albicans* in blood plasma and in whole blood. Once MB is the mostly used PS to disinfect plasma, its efficacy was also evaluated for comparation;
- Evaluate if the concentrates of red blood cells are affected by the PS concentration and by plasma after being submitted to aPDT treatment.

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# Photodynamic Inactivation of *Candida albicans* in Blood Plasma and Whole Blood

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# Chapter II

## 1. Abstract

The few approved disinfection techniques for blood derivatives promote damage in the blood components, representing risks for the transfusion receptor. Antimicrobial photodynamic therapy (aPDT) seems to be a promising approach for the photoinactivation of pathogens in blood, but only three photosensitizers (PSs) have been approved, methylene blue (MB) for plasma and riboflavin and amotosalen for plasma and platelets. In this study, the efficiency of the porphyrinic photosensitizer Tri-Py(+)-Me and of the porphyrinic formulation FORM was studied in the photoinactivation of *Candida albicans* in plasma and in whole blood and the results were compared to the ones obtained with the already approved PS, MB. The results show that FORM and Tri-Py(+)-Me are promising PSs to inactivate *C. albicans* in plasma**.** Although in whole blood the inactivation rates obtained were higher than the ones obtained with MB, further improvements are required. None of these PSs had promoted hemolysis at the isotonic conditions when hemolysis was evaluated in whole blood and after the addition of treated plasma with these PSs to concentrates of red blood cells

## 2. Introduction

Human blood is a key tissue responsible for transporting vital molecules like oxygen, carbon dioxide and glucose to the different parts of the body. This circulating fluid is composed of plasma, erythrocytes or red blood cells, leukocytes or white blood cells and thrombocytes or platelets [1-3]. Plasma constitute about 55% of whole blood, the red blood cells about 45% and white blood cells less than 1% [3].

Blood transfusions are usually required by patients with hemorrhagic diseases, anemia and after major surgeries, etc. This medical procedure can occur by whole blood transfusion and more commonly, by transfusions of blood components such as erythrocytes, plasma or platelets. In some cases, multiple blood transfusions are required to meet the body's needs. Transfusions of erythrocytes concentrates are used in hypoxia conditions by blood loss after trauma or surgery [4-6]. These erythrocytes concentrates can be stored for 35 days at 2–6 °C in red cell preservation solutions [5,7]. Platelet transfusions are used for preventing or treating bleeding in patients with thrombocytopenia or abnormalities of platelet function [8,9]. It can be concentrated from plasma or by plateletpheresis from a single donor and stored at 22 °C for 5 days [10].

Plasma transfusions are required to correct deficiencies of clotting factors, for which a specific concentrate is not available, in patient in severe bleeding [4]. Plasma can be frozen promptly, stored at −18 °C for 5 years and could be defrosted before use.

To ensure the safety of transfusions in the blood collection, several procedures are adopted, including donor screening, specific serological and nucleic acid testing and transfusion hemovigilance. Despite the measures already adopted, microbial infections are yet transmitted through blood products transfusion [5,11,12] causing diseases in the blood receptor [5,13,14]. In the US, bacterial contamination is considered the second most common reason of death from a transfusion, resulting in morbidity and mortality from 100 to 150 transfused individuals each year [15]. Between 2010 and 2013, 111 transfusion-transmitted infections (TTIs) were detected in the European Union being 66% bacterial, 32% viral and less than 3% parasites [16,17]. Due to the combination of plasma fractions collected from different donors, the infections rates are more noteworthy in transfusions involving blood plasma or products derived from plasma [5,7]. It is in this context that the disinfection of blood and blood products assume great importance.

The conventional treatments used to inactivate pathogenic microorganisms in blood and blood products were developed essentially to inactivate virus [5,7,18]. The World Health Organization (WHO) recommends the screening for the presence of Human Immunodeficiency Virus (HIV), Hepatitis B Virus (HBV), Hepatitis C Virus (HCV) and the bacterium *Treponema pallidum* (causative agent of syphilis), for all blood donations [19]. However, the collected blood can contain other pathogenic agents like fungi [20], parasites [21,22] and several bacteria [5,15,23,24], which come especially from the intestine or from pre-symptomatic infections or coming from exogenous microorganisms, such as from skin. Nowadays, there are some available methods for the reduction of pathogens in blood products, but they are approved essentially for plasma.

The most generalized method combines the use of tri(*n*-butyl)phosphate and detergent Tween 80 and can be only used in plasma or protein concentrates. Due to the negative effects that these chemicals promote in the erythrocyte membranes and platelets, they must be removed after treatment [7,25–27]. The use of ultraviolet (UV) light is also considered and this methodology is essentially applied in plasma and platelet disinfection. The irradiation causes damage to the microbial genetic material avoiding its replication;

however, this technique produces free radicals that are extremely cytotoxic [5,7,28,29]. Other processes used in blood purification such as chromatographic techniques using specific antibodies adsorbed and even physical methods to remove extracellular pathogens, like nanofiltration or cell washing are also in use for plasma [5,7,30]. However, they cannot be applied in concentrated platelets and erythrocytes since cell membranes can bind non-specifically to the antibodies and intracellular pathogens are not filtrated or wash removed by these techniques [5,7,30].

Another approach for blood disinfection is the antimicrobial photodynamic therapy (aPDT). In this therapeutic approach the combination of a photosensitizer (PS) and visible light in the presence of molecular oxygen, produces highly toxic oxygen species (ROS), such as singlet oxygen ($^1O_2$), hydrogen peroxide ($H_2O_2$), superoxide ($O_2^{\bullet-}$) and hydroxyl radicals ($OH^{\bullet}$) [31,32]. These species are responsible for the irreversible oxidation of vital constituents of microorganisms, causing their death [32,33]. Nowadays, only three Ps [amotosalen (a psolaren), riboflavin (or vitamin B2) and methylene blue (MB)] are approved for blood disinfection and these can only be used for plasma and platelets treatment. The aPDT with amotosalen and riboflavin requires the use of UV (UVA) and UV (UVB), respectively, which may lead to the formation of harmful free radicals. Despite the radical species formed affect plasma proteins and platelets, the amotosalen and riboflavin were approved in Europe for platelets and plasma disinfection [12,34,35]. Contrarily, the aPDT with MB uses visible light instead of UV light and it is also approved for pathogen inactivation in plasma units [7,36]. In this case most enveloped viruses are inactivated, but non-enveloped viruses, intracellular viruses, protozoa, fungi and bacteria remain unaffected [7,37]. Due to the poor intracellular uptake of MB, this photosensitizer cannot inactivate intracellular pathogens [5,12,38,39]. Although MB may interact with clotting factors and most noticeably fibrinogen and factor VIII with a loss of approximately 30%, no relevant side effects were detected in patients transfused with MB-treated plasma [12,40,41]. Nevertheless, some doubts have arisen about the efficacy of MB in the treatment of plasma when used as a replacement solution for plasma exchange in the treatment of patients suffering from thrombotic thrombocytopenic purpura [36,42]. Although it is approved for the disinfection of plasma in several European countries, in France it was removed from the market due to allergic reactions detected in a few patients that received plasma treated with MB [36].

In what concerns to the disinfection of whole blood and erythrocytes concentrates, there is a great difficulty in the development of effective methodologies due to the complexity and sensitivity of the matrix. A recent study shows that the use of S-303, a positively charged synthetic alkylating agent, can disrupt the pathogen genetic material in erythrocytes [12,13,43,44]. Besides this promising study, no well-established method for whole blood disinfection was approved.

Having in mind the lack of more efficient methods for the inactivation of pathogens in blood products, the combination of MB as a PS and visible light based in the photodynamic therapy effect seems to be the more consensual method for the pathogenic inactivation in plasma. In fact, aPDT represents a non-antibiotic approach, that has been shown to be effective in the photoinactivation of bacteria, viruses, fungi, and protozoa [45]. aPDT action is multi-target, with the great advantage over traditional approaches, which means that this therapy acts on a variety of biochemical targets (extra and intracellular structures) and therefore prevents the development of microbial resistance mechanisms [46]. In fact, repeated photosensitization does not induce resistance in microorganisms [33,45]. However, fungi are more complex microorganisms and therefore become more challenging targets than viruses and bacteria. Fungi inactivation seems to be less dependent on the binding of PS to cells. In the case of fungi, the free PS induces some initial changes in the plasmatic membrane and then penetrate in the cell, causing more extensive damage in more complex subcellular structures such as mitochondria or nucleus [45,47].

There are few studies reporting fungal infections transmitted by blood transfusions. In 2011, a statistical study analyzed 86 patients with severe abdominal sepsis and severe pancreatitis, in which 23% of these patients were colonized by *Candida* and 8% of the patients developed candidiasis after transfusion of at least four volumes of red blood cells [48]. *C. albicans* is a commensal fungus that can be isolated from the gastrointestinal tract, oral and vaginal mucosa of healthy individuals, existing in balance with the bacterial flora and host immune system [49]. This fungus presents high ability to survive and proliferate in adverse environments with drastic changes in oxygen, carbon dioxide, nutrients, pH, osmolality and temperature [47]. Another important feature of *C. albicans* is its ability to form biofilms, that are a problem in medical practice because they can be formed in artificial heart valves and dentures, presenting resistance to various antifungal agents currently used

in clinical practice, including amphotericin B and fluconazole, and have multiple mechanisms of resistance [50]. In disseminated candidiasis in individuals with the compromised immune system, *C. albicans* gains direct access to the bloodstream with a mortality rate of approximately 40% [51,52].

*C. albicans* has been shown to be susceptible to aPDT in their planktonic [53,54] and biofilms forms [54,55]. Moreover, this therapy appears to prevent the formation of biofilms by reducing their adhesion capacity [56]. The haematoporphyrin [57], Photofrin [58,59], *meso*-tetrakis(*N*-methyl-6-quinolinyl)-substituted porphyrins and chlorins [60], cationic derivatives of *meso*-tetrapyridylporphyrin [61], porphyrin derivatives bearing a fluconazole unit [62] and ALA [63] are some of the PS used in vitro assays that efficiently inactivate *C. albicans*.

In this work, we report the study of the photodynamic effect of 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin tri-iodide (Tri-Py(+)-Me) and a PS formulation (FORM), based on a non-separated mixture of five cationic *meso*-tetraarylporphyrins (**Figure 2.1**), in the photoinactivation of *C. albicans* in blood plasma and in whole blood. The way that concentrates of red blood cells are affected by the PS concentration and by plasma after being submitted to aPDT was also evaluated. All the results were compared with the ones achieved with the approved MB. The selected porphyrinic PSs have already proved their efficiency in the photoinactivation of *Escherichia coli* [5,64,65], *Pseudomonas syringe* [66], *Staphylococcus aureus* [5,64,65], and *C. albicans* [54]. FORM has been considered an excellent alternative to the highly efficient constituents, Tri-Py(+)-Me and Tetra-Py(+)-Me, since production costs were reduced significantly due to its use [54,64,65]. Moreover, Tri-Py(+)-Me was recently described as a potential PS for the inactivation of *E. coli* and *S. aureus* in blood plasma and whole blood [5]. In this report, this PS had promoted the total inactivation of *S. aureus* in blood plasma and *c.a.* of 4 $\log_{10}$ in *S. aureus* viability in whole blood. In the case of *E. coli*, a reduction in the survival of this bacterium of *c.a.* 6 $\log_{10}$ and 5 $\log_{10}$ was achieved for plasma and whole blood, respectively. Furthermore, it was demonstrated that the Tri-Py(+)-Me, at 5.0 and 10 μM, does not promote hemolysis in isotonic conditions (0.9% NaCl) [5].

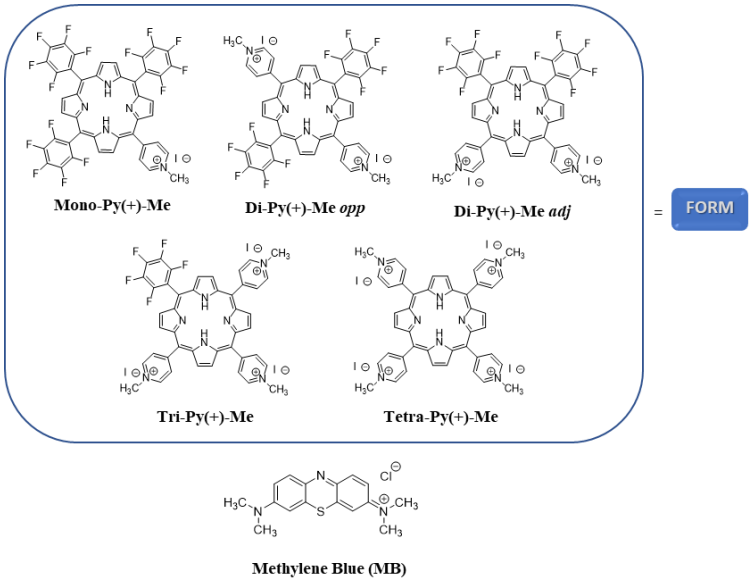


***Figure 2.1:*** *Structures of the photosensitizers (PSs) used in this study to photoinactivate C. albicans.*

## 3. Materials and Methods

### 3.1. Blood Samples

Human blood samples were voluntarily provided by Avelab clinical laboratory (Aveiro, Portugal). The blood tubes provided contains whole blood but also 5.4 mg of an anticoagulant-EDTAk$_3$ prefacing a final volume of 3 mL (BD Vacutainer®, Becton Dickinson, Plymouth, UK). The blood samples were used for up to 5 days after being received and were stored under appropriate conditions. Plasma was obtained after centrifugation of whole blood at 3500 rpm (Heraeus Megafuge 16R, Waltham, USA) for 5 min.

Fresh human blood samples (<24 h) used for erythrocyte osmotic fragility assays were kindly provided by volunteers and collected at CMM- Aveiro Medical Center. The blood tubes contain 16.2 mg of EDTAk$_3$ prefacing a final volume of 9 mL (Vacumed®, Torreglia, Italy).

### 3.2. Characterization of microbial strains and culture conditions

The yeast *C. albicans* (ATCC 10231) was maintained on Yeast Extract Glucose Chloramphenicol Agar (YGCA, Liofilchem, Roseto degli Abruzzi, Italy) at 4 °C. Before each assay, a colony was transferred to 20 mL of YG [Yeast extract (5 g/L) + Glucose (10

g/L)] and incubated for 24 h at 37 °C with constant stirring (120 rpm). Then 200 μL aliquots were transferred to new 20 mL YG and incubated at the previous growth conditions in order to reach the stationary phase, corresponding to a concentration of $10^6$–$10^7$ colony forming units per mL ($CFU \cdot mL^{-1}$).

### 3.3. Light sources

The efficiency of the PSs in PBS was evaluated by exposing the samples and controls of a set of 13 white fluorescent lamps (PAR radiation, OSRAM 21 lamps of 18 W each, 380–700 nm) for a maximum irradiation period of 270 min with an irradiance of 2.5 $mW \cdot cm^{-2}$.

In aPDT assays for plasma and whole blood, the samples and controls were irradiated with a compatible fiber optic probe attached to a 250 W quartz/halogen lamp (LUMACARE model 122, Newport Beach, California, USA) with an irradiance of 150 $mW \cdot cm^{-2}$ for a maximum irradiation period of 270 min. All the irradiations were measured with a Coherent FieldMaxII-Top combined energy meter (COHERENT, Santa Clara, USA).

### 3.4. Photosensitizers

Stock solutions of FORM and Tri-Py(+)-Me were prepared in DMSO at a concentration of 500 μM and stored in the dark. Stock solution of MB was prepared in PBS at 500 μM and stored in the dark. All photosensitizers were sonicated for 30 min before each assay (ultrasonic cleaner, Nahita 0.6 L, 40 kHz, GT SONIC Technology, Guangdong, China).

The porphyrins 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin tri-iodide (Tri-Py(+)-Me), 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (Tetra-Py(+)-Me) and the formulation (FORM) [a mixture of non-separated porphyrins: 5-(1-methylpyridinium-4-yl)-10,15,20-tris(pentafluorophenyl)-porphyrin mono-iodide (Mono-Py(+)-Me) (19%), 5,15-bis(1-methylpyridinium-4-yl)-10,20-bis(pentafluorophenyl)porphyrin di-iodide (Di-Py(+)-Me *opp*) and 10-bis(1-methylpyridinium-4-yl)-15,20-bis(pentafluorophenyl)porphyrin di-iodide (Di-Py(+)-Me *adj*) (20%), Tri-Py(+)-Me (44%) and Tetra-Py(+)-Me (17%)] were synthetized according with the literature and their structures are presented in **Figure 2.1**

[65,66,67]. The UV-Vis spectrum of these photosensitizers was already reported in the literature [64,65].

### 3.5. Antimicrobial Photodynamic assays

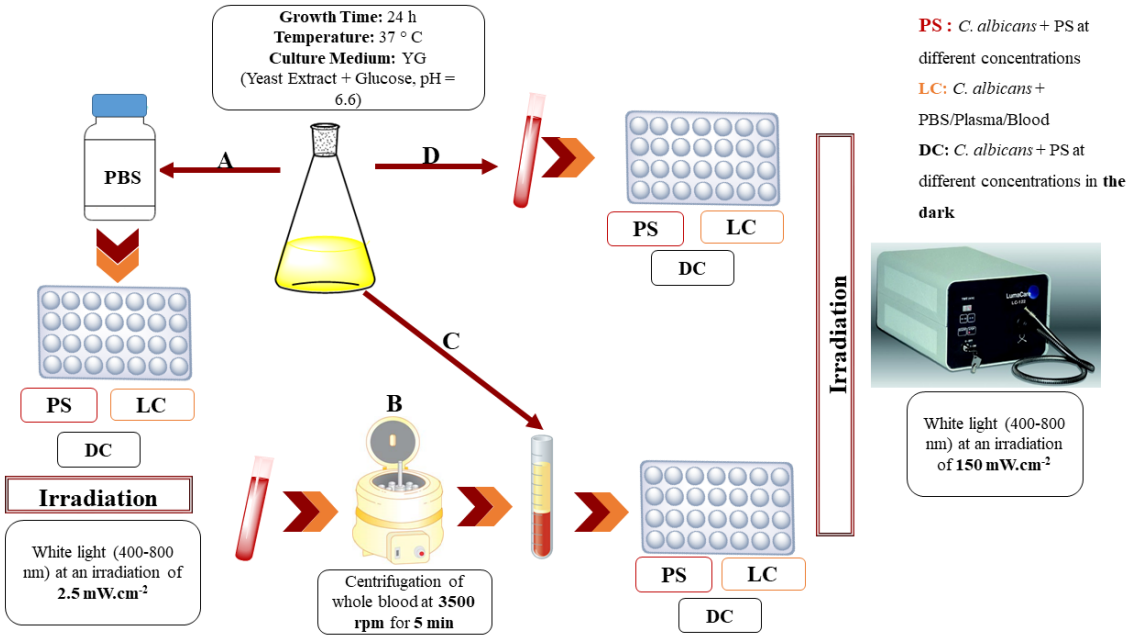


***Figure 2.2:*** *Schematic representation of the photodynamic assays.*

The *C. albicans* culture, after reaching the stationary phase was diluted (1:10) in the selected matrix: PBS (**Figure 2.2A**), plasma (**Figure 2.2C**), and whole blood (**Figure 2.2D**). Then, the resulting suspension was distributed to the wells of a 12-well plate. Plasma was obtained by centrifugation of whole blood at 3500 rpm for 5 min (Heraeus Megafuge 16R, Waltham, USA) (**Figure 2.2B**). Each PS was then added to the samples making a final concentration of 5.0 μM in PBS, 5.0 μM and 10 μM in plasma, and finally 10 μM in whole blood. Light and dark controls were also carried out simultaneously with the aPDT procedure: the light controls (LC) comprised a *C. albicans* suspension and the dark control (DC) comprised the fungus suspension incubated with the PSs at the higher concentration tested protected from light. To promote the interaction of the photosensitizer with the fungus, samples and controls were incubated before irradiation for 10 min in PBS and 30 min in plasma and whole blood under constant agitation in the dark. Increased incubation time for plasma and whole blood was required due to the protein and cellular complexity of these matrices, which made the interaction more challenging.

Samples and light controls (LC) were irradiated under the conditions described above while the dark control was protected from light during treatment. The samples and each control were kept in agitation at a controlled temperature of 25 °C. The photoinactivation capacity of each PS was evaluated by quantifying the number of CFU per volume ($CFU.ml^{-1}$). Aliquots of samples and of each control were taken at time 0 min (after incubation time) and at different irradiation times (15, 30, 60, 90, 180, and 270 min). After this, serial dilutions were made and finally plated in YGCA and incubated for 4 days at 37 °C. The same conditions were used in all experiments, for each condition three independent assays with two replicates each were performed.

### 3.6. Evaluation of aPDT and DMSO effect on erythrocyte osmotic fragility

The effect of aPDT on erythrocyte osmotic fragility was evaluated using 5.0, 10, and 20 μM of FORM and MB. These assays were already described in the literature [5,68]. The erythrocyte osmotic fragility was evaluated before (incubation time) and after aPDT treatment; firstly, the PS was added to samples and dark controls, and the resulting samples and controls were incubated for 30 min in the dark. Thus, samples and light controls were irradiated at 150 $mW.cm^{-2}$. Aliquots of 20 μL of each samples and controls were added to eppendorf tubes with 1980 μL of NaCl solutions (0, 0.1%, 0.3%, 0.5%, 0.7%, 0.9%) and were incubated at 25 °C for 30 min with constant stirring. Finally, all samples were centrifuged at 3500 rpm for 10 min (Gyrozen 1730R, Gimpo, Korea), following were collected the supernatants resulting from this centrifugation and their optical densities were measured. The optical density of the supernatant was determined spectrophotometrically (Multiskan FC, Thermo Scientific, Waltham, USA) at 540 nm, the wavelength recommended for evaluating the amount of hemoglobin in solution. Hemolysis was represented in percentage by considering the optical density value of distilled water solution (0% NaCl) as 100%. A similar procedure was performed but using different percentages of DMSO (1%, 2%, 3%, and 4%).

### 3.7. Evaluation of erythrocyte osmotic fragility after the addition of the treated plasma with FORM, Tri(+)-Py-Me and MB to the to the concentrated erythrocytes

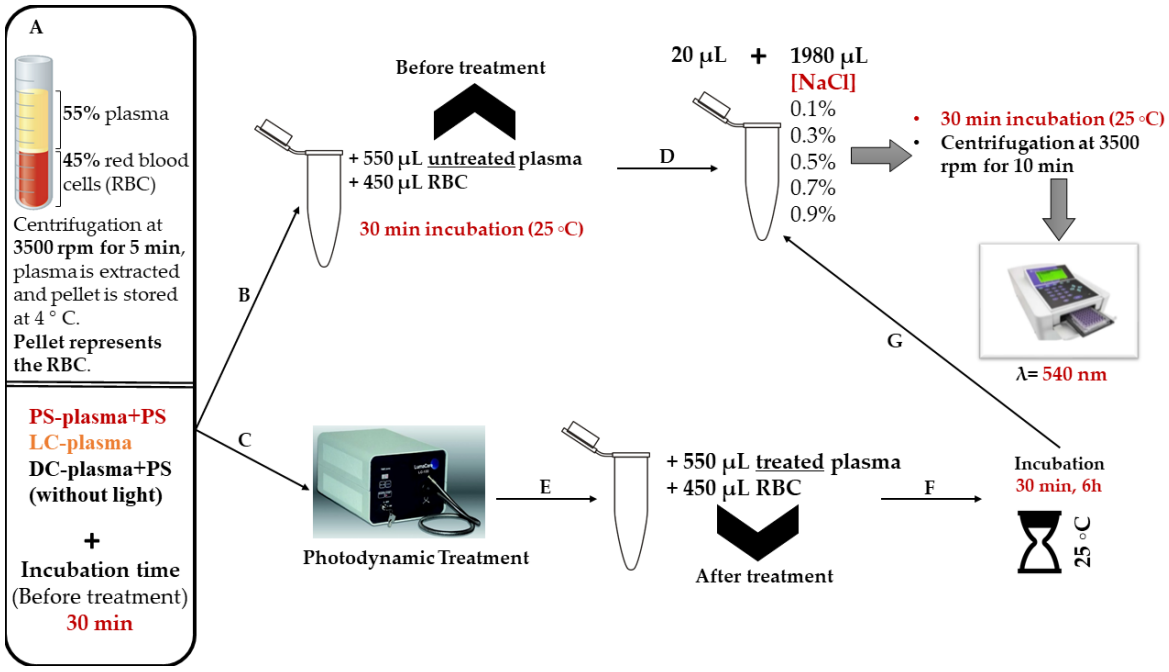


***Figure 2.3:*** *Schematic representation of the experimental protocol to evaluate the erythrocyte osmotic fragility after the addition of the treated plasma with FORM, Tri-Py(+)-Me and MB to the concentrated erythrocytes.*

These assays were performed in order to evaluated possible negative effects in erythrocyte membranes that aPDT-treated plasma transfusion with FORM, Tri-Py(+)-Me, and MB can cause in the receptor. For this purpose, the erythrocyte osmotic fragility induced by untreated (after incubation time) and treated (after aPDT) plasma was evaluated. Initially the whole blood was centrifuged at 3500 rpm for 5 min (Heraeus Megafuge 16R, Waltham, USA) to extract the plasma, while the centrifugation pellet containing the red blood cells was stored at 4 °C. After collecting the plasma, the PS was added to samples and dark controls, and both samples and controls were incubated for 30 min in the dark (**Figure 2.3A**). Then, the samples and light controls were irradiated (150 mW.cm$^{-2}$) and the dark controls kept in the dark (**Figure 2.3C**). The treated plasma was incubated with the red blood cells concentrates for 30 min and 6 h and so the effect of treated plasma on red blood cells at short and long term was evaluated (**Figure 2.3F**). For this, the interaction of treated plasma and the red blood cells concentrates was prepared in eppendorf tubes at a final volume of 1 mL in a proportion of 55% plasma/45% red blood cells (**Figure 2.3B** and **E**) [3]. Each eppendorf

tube was incubated at 25 °C for 30 min and 6 h with constant stirring. All of the remaining steps of the protocol were the same as the ones performed in the previous section.

### 3.8. Statistical

At least three independent experiments with two replicates per assay for each condition were done. The statistical analysis was performed with GraphPad Prism (GraphPad Software, San Diego, USA). Normal distributions were checked by the Kolmogorov–Smirnov test and the homogeneity of variance was verified with the Brown Forsythe test. ANOVA and Dunnet's multiple comparison tests were applied to assess the significance of the differences between the tested conditions. A value of $p < 0.05$ was considered significant.

## 4. Results

### 4.1. Photodynamic efficiency of FORM, Tri-Py(+)-Me, Tetra-Py(+)-Me and MB in the inactivation of *C. albicans* in PBS

**Figure 2.4** presents the photodynamic inactivation profile of *C. albicans* in PBS in the presence of FORM, Tri-Py(+)-Me, Tetra-Py(+)-Me, and MB at 5.0 μM when irradiated with white light (380–700 nm) at 2.5 $mW.cm^{-2}$. The results showed that the porphyrin derivatives are effective in the photodynamic inactivation of *C. albicans*, promoting a decrease in the survival of the fungus until the detection limit was reached after 30, 180, and 270 min of irradiation, for FORM, Tri-Py(+)-Me, and Tetra-Py(+)-Me , respectively. Its noteworthy the results achieved with FORM, being the PS with higher (ANOVA, $p < 0.05$) rate of photoinactivation of *C. albicans* in the shortest light exposure time. Although effective against this fungus, its constituents, Tri-Py(+)-Me and Tetra-Py(+)-Me , caused a decrease of c.a. 0.6 $\log_{10}$ (ANOVA, $p < 0.05$) in the survival of *C. albicans* after 30 min of irradiation. The MB (used as PS reference), was less effective, causing a decrease of 0.8 $\log_{10}$ in *C. albicans* survival after 270 min of irradiation.

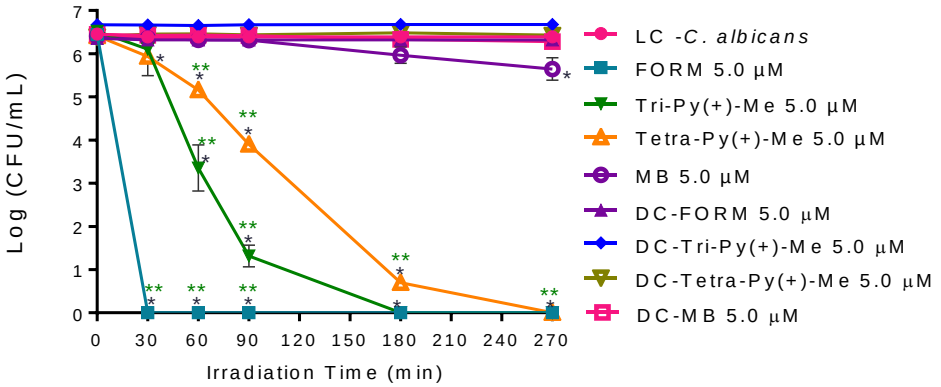


***Figure 2.4:*** *Photodynamic inactivation of C. albicans in the presence of FORM, Tri-Py(+)-Me, Tetra-Py(+)-Me, and MB at 5.0 µM in PBS and irradiated with white light (380–700 nm) at 2.5 mW.cm$^{-2}$. Values represent the average of three independent experiments with two replicates each; error bars indicate the standard deviation. Lines just combine the experimental points. * $p < 0.05$ (relatively to the LC); ** $p < 0.05$ (relatively to MB).*

### 4.2. Evaluation of aPDT effect on erythrocyte osmotic fragility

In order to choose a safe concentration of each PS to be used in the aPDT of *C. albicans* in blood plasma and whole blood, the erythrocyte osmotic fragility was assessed at different concentrations of each PS (5.0, 10, and 20 µM) before (0 min) and after aPDT treatment (90 min). Concentrations that did not promote hemolysis after the aPDT protocol were used in the photoinactivation of *C. albicans* in plasma and in whole blood. Since Tetra-Py(+)-Me was the least efficient porphyrinic PS in the *C. albicans* photoinactivation in PBS, this PS was not included in the following studies.

Thus, blood samples before and after exposure to aPDT protocol (incubation with each PS concentration followed by irradiation with white light at 150 mW.cm$^{-2}$ for 90 min) were added to tubes containing increasing concentrations of sodium chloride (NaCl) solution (0, 0.1, 0.3, 0.5, 0.7, and 0.9%) at pH 7.4 and the hemoglobin was spectrophotometrically quantified. The results of the erythrocyte osmotic fragility of Tri-Py(+)-Me were already reported and had shown that this PS did not promote significant (ANOVA, $p > 0.05$) erythrocytes hemolysis after aPDT at 5.0 and 10 µM using a non-stress (isotonic) condition (0.9% NaCl) [5].

**Figure 2.5** shows the results achieved for osmotic erythrocyte fragility promoted by FORM at 5.0, 10, and 20 μM before and after the aPDT protocol. In the isotonic solution (0.9% NaCl) and for the concentration of 5.0 μM, no significant (ANOVA, $p > 0.05$) erythrocytes hemolysis were observed. The same profile was attained for the NaCl solution at 0.7%. However, when submitted to a 0.5% NaCl solution, FORM at 5.0 μM endorsed hemolysis rates of 38% ($p < 0.05$) and 51% (ANOVA, $p < 0.05$) before and after aPDT, respectively. In this case, no significant differences were observed between dark control and irradiated samples treated with FORM. Also, for FORM at 10 μM, observed conditions were not considered significant (ANOVA, $p > 0.05$) for erythrocytes hemolysis under non-stress (isotonic) conditions, both before and after aPDT protocol. However, for the NaCl solution at 0.7%, hemolysis rates of 20% (ANOVA, $p < 0.05$) and 42% (ANOVA, $p < 0.05$) were observed before and after irradiation. For the NaCl solutions at 0.5% and at lower concentrations, the hemolysis observed increases (higher than 64%), which was significantly different from the hemolysis rate achieved in the light control (ANOVA, $p < 0.05$). Also in this case, no significant differences were observed between dark control and irradiated samples treated with FORM. When the osmotic erythrocyte fragility was studied with FORM at 20 μM, high hemolysis rates were observed in all NaCl solutions, even before the aPDT protocol. For example, in the isotonic solution (0.9% NaCl), 45% (ANOVA, $p < 0.05$) and 82% (ANOVA, $p < 0.05$) of hemolysis was observed, before and after aPDT, respectively. In fact, after irradiation, in all the NaCl solutions the observed hemolysis was higher than 80%. Once again, no significant differences were observed between dark control and irradiated samples treated with FORM.

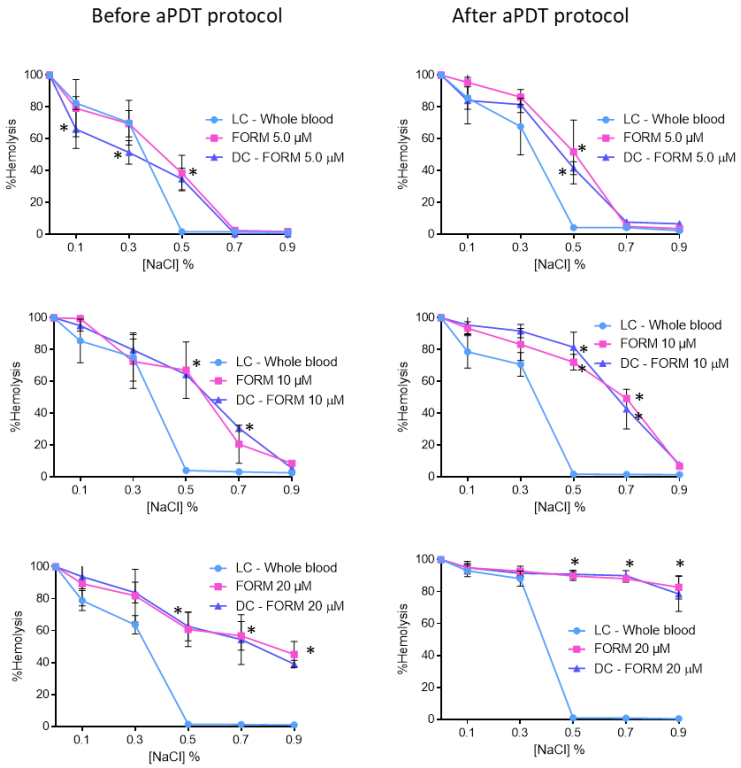


***Figure 2.5**: Erythrocyte osmotic fragility before and after aPDT treatment under white light (380–700 nm) at an irradiance of 150 mW.cm$^{-2}$, with FORM at 5.0, 10, and 20 µM. Light control (erythrocytes under light) and dark control (erythrocytes incubated with FORM without irradiation) were included. Values represent the average of three independent experiments; error bars indicate the standard deviation. Lines just combine the experimental points. * $p < 0.05$ (relatively to the LC).*

The results of the osmotic erythrocyte fragility tests with MB at 5.0, 10, and 20 µM are presented in **Figure 2.6**. In all solutions with percentages of NaCl equal to 0.5% or higher, no significant hemolysis was observed. For MB at 5.0 µM, 10, and 20 µM after the irradiation protocol, it was possible to observe hemolysis rates of 71% (ANOVA, $p < 0.05$), 92% (ANOVA, $p < 0.05$), and 93% (ANOVA, $p < 0.05$) for the 0.3% NaCl solution, respectively. In all cases no significant differences were observed between dark control and irradiated MB.

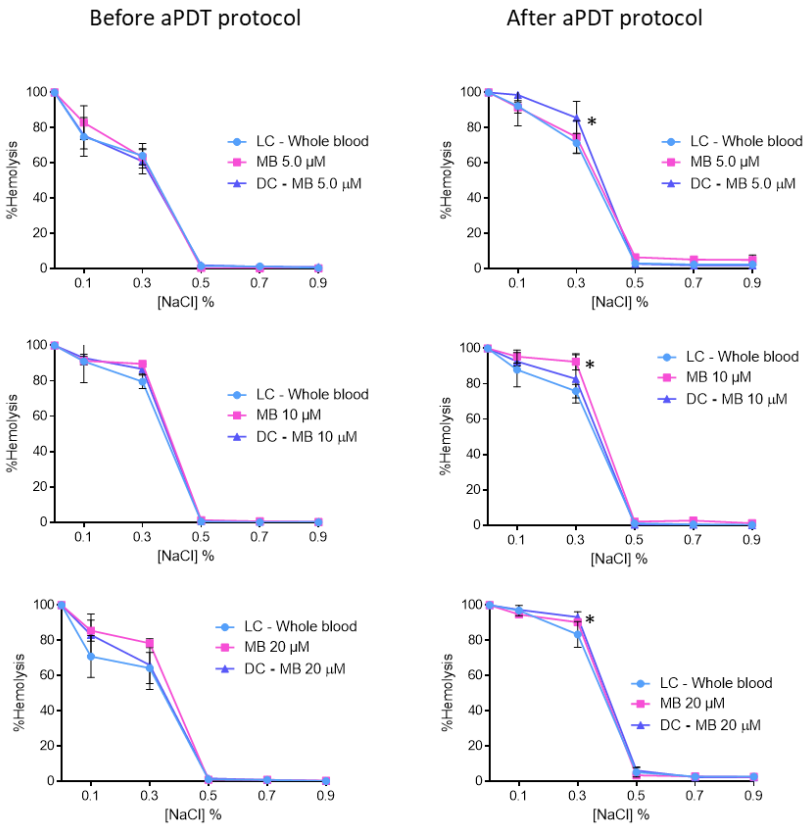


***Figure 2.6**: Erythrocyte osmotic fragility before and after aPDT treatment under white light (380–700 nm) at an irradiance of 150 mW.cm$^{-2}$, with MB at 5.0, 10, and 20 µM. Light control (erythrocytes under light) and dark control (erythrocytes incubated with MB without irradiation) were included. Values represent the average of three independent experiments; error bars indicate the standard deviation. Lines just combine the experimental points. * $p < 0.05$ (relatively to the LC).*

Contrary to the MB stock solution, which was prepared in PBS, the porphyrinic stock solutions were prepared in DMSO (FORM and Tri-Py(+)-Me) due to their limited solubility in aqueous solutions. So, the osmotic erythrocyte fragility promoted by DMSO was accessed in the same percentages used in the assays with each porphyrinic PS. Thus, 1%, 2% and 4% of DMSO were added to blood samples and submitted to the aPDT protocol (irradiation with white light at 150 mW·cm$^{-2}$ for 90 min). The samples were added to tubes containing increasing concentration of NaCl solution (0%, 0.1%, 0.3%, 0.5%, 0.7%, and 0.9%) at pH 7.4 and the hemoglobin was quantified before and after the irradiation procedure. The results obtained are presented in **Figure 2.7**. As it is possible to observe, samples with 1% and 2% of DMSO did not promote significant hemolysis in the 0.7% and 0.9% NaCl solutions either before and after the aPDT protocol. However, in the solutions with lower percentages of NaCl the hemolysis rates promoted by these percentages of DMSO increased; before the irradiation, the erythrocyte solutions with 1% and 2% of DMSO suffered a hemolysis rate

of 14 (ANOVA, $p < 0.05$) and 22% (ANOVA, $p < 0.05$), respectively, in the NaCl solution at 0.5%. After the aPDT protocol, these conditions promoted higher hemolysis: 40 (ANOVA, $p < 0.05$) and 45% (ANOVA, $p < 0.05$), for 1% and 2% of DMSO, respectively. When the osmotic erythrocyte fragility was studied with 4% of DMSO high hemolysis rates were observed in all NaCl solutions, even before the irradiation protocol, with the exception of the isotonic solution before the aPDT protocol, where no hemolysis was observed. After the irradiation procedure and in NaCl solutions at 0.3%, 0.5%, 0.7%, and 0.9%, the hemolysis rates were 45% (ANOVA, $p < 0.05$).

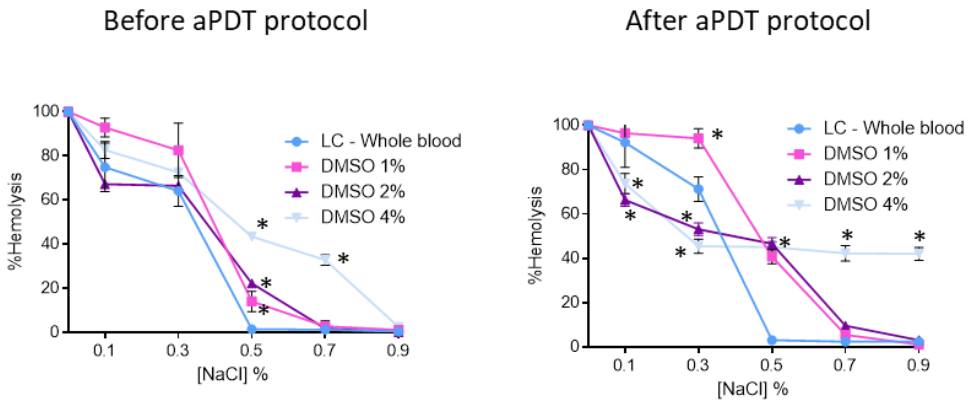


***Figure 2.7:*** *Erythrocyte osmotic fragility before and after aPDT treatment under white light (380–700 nm) at an irradiance of 150 mW.cm$^{-2}$, with 1%, 2%, and 4% of DMSO. Light control (erythrocytes under light) was included. Values represent the average of three independent experiments; error bars indicate the standard deviation. Lines just combine the experimental points. * $p < 0.05$ (relatively to the LC).*

### 4.3. aPDT of *C. albicans* in blood plasma using FORM, Tri-Py(+)-Me and MB

After the erythrocyte osmotic fragility studies, the PS concentrations that did not promoted significant hemolysis in the isotonic conditions were used in the inactivation of *C. albicans* in blood plasma. Thus, FORM, Tri-Py(+)-Me and MB at 5.0 and 10 μM were tested against the fungus strain in plasma applying the same irradiation conditions used in the erythrocyte osmotic fragility studies (white light at 150 mW.cm$^{-2}$) and the results are presented in **Figure 2.8**.

The results showed that FORM at 5.0 and 10 μM was capable to photoinactivate *C. albicans* in blood plasma. When compared with the light control (LC), FORM had promoted a decrese of 1.2 $\log_{10}$ ($p < 0.05$) at 5.0 μM and 1.7 $\log_{10}$ ($p < 0.05$) at 10 μM in the fungus survival after 270 min of light irradiation. No effects in the *C. albicans* survival were observed in dark controls (DC) as well in the DMSO control (DMSO CT).

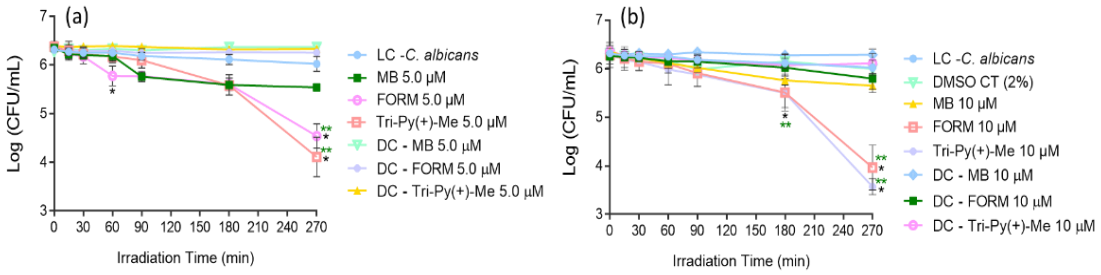


***Figure 2.8**: Photodynamic inactivation of C. albicans in the presence of FORM, Tri-Py(+)-Me, and MB at 5.0 (a) and 10 µM (b) in blood plasma and irradiated with white light at at 150 mW·cm$^{-2}$. Values represent the average of three independent experiments; error bars indicate the standard deviation. Lines just combine the experimental points. * $p < 0.05$ (relatively to the LC); **$p < 0.05$ (relatively to MB).*

When Tri-Py(+)-Me was used as PS in the photoinactivation of *C. albicans* in blood plasma (**Figure 2.8**), the photodynamic profile attained was similar to the one observed for FORM, although the decrease in the fungus survival rate was significantly higher. As is possible to observe, Tri-Py(+)-Me at 10 µM and after 180 min of irradiation had promoted a decrease of 0.6 $\log_{10}$ (ANOVA, $p < 0.05$) when compared with LC. After 270 min of aPDT, a decrease of 1.9 and 2.5 $\log_{10}$ (ANOVA, $p < 0.05$) in the *C. albicans* survival was achieved at 5.0 and 10 µM of Tri-Py(+)-Me, respectively. As in the previous case, no significant effects in the *C. albicans* survival were observed in dark controls (DC).

The reference PS MB was shown to be the least efficient PS in the photoinactivation of *C. albicans* in blood plasma, causing a tiny decrease in fungus survival: 0.5 and 0.4 $\log_{10}$ (ANOVA, $p < 0.05$), for 5.0 and 10 µM, respectively, after 180 min of aPDT (**Figure 2.8**). It is important to note that the photodynamic inactivation profile remained almost constant between 90 and 270 min of irradiation. It was also observed that the blue color of the plasma solution, which was present in the beginning of the aPDT protocol, disappeared throughout the photodynamic process. Also in this case, no significant effects in the *C. albicans* survival were observed in dark controls (DC).

### 4.4. aPDT of *C. albicans* in whole blood using FORM and Tri-Py(+)-Me

The most promising PSs in the photoinactivation of *C. albicans* in blood plasma, FORM and Tri-Py(+)Me at 10 μM, were used to photoinactivate this fungus in whole blood artificially contaminated. The results presented in **Figure 2.9**, show that the photoinactivation of *C. albicans* in the presence of FORM started after 180 min of irradiation, causing a decrease of 0.7 $\log_{10}$ (ANOVA, $p < 0.05$) in its survival after 270 min of treatment. In the case of Tri-Py(+)-Me (**Figure 2.9**), the decrease in *C. albicans* survival began at 90 min of irradiation, and reached a decrease in the fungus survival of 0.6 $\log_{10}$ (ANOVA, $p < 0.05$) and 0.7 $\log_{10}$ (ANOVA, $p < 0.05$) after 180 min and 270 min of aPDT protocol, respectively. In both cases, no significant reduction on the *C. albicans* survival was achieved in the dark controls.

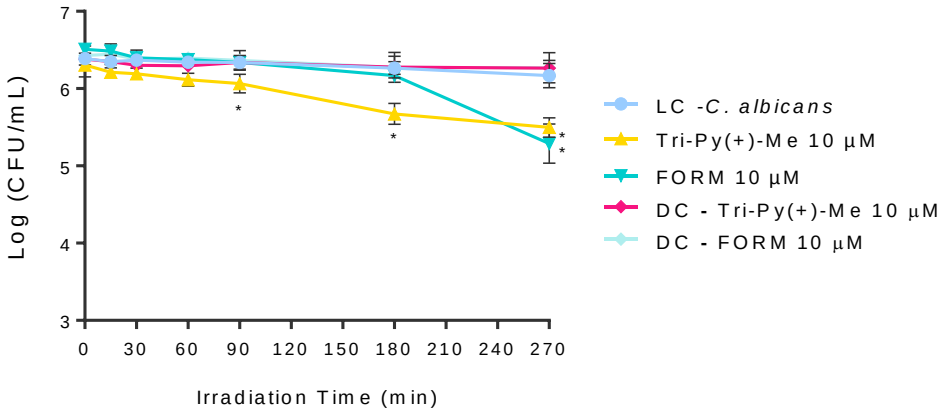


***Figure 2.9:*** *Photodynamic inactivation of C. albicans in the presence of FORM and Tri-Py(+)-Me at 10 μM in whole blood and irradiated with white light at at 150 mW.cm$^{-2}$. Values represent the average of three independent experiments; error bars indicate the standard deviation. Lines just combine the experimental points. * p < 0.05 (relatively to the LC).*

### 4.5. Evaluation of erythrocyte osmotic fragility after the addition of the treated plasma with FORM, Tri-Py(+)-Me and MB to the concentrated erythrocytes

Having in mind the potential application of FORM and Tri-Py(+)-Me as PSs in the *C. albicans* inactivation in blood plasma, the erythrocyte osmotic fragility after the addition of treated plasma to the concentrated erythrocytes was assessed. Thus, blood plasma solutions were submitted to aPDT protocol (described for the blood plasma photodynamic assays) in the presence of FORM, Tri-Py(+)-Me, and MB at 5.0 and 10 μM. After aPDT,

aliquots of treated plasma were added to the concentrated erythrocytes and then osmotic fragility was assessed after 30 min and 6 h of incubation in the dark (see **Figure 2.3**).

The results had shown that plasma treated with FORM at 5.0 μM did not promoted significant erythrocyte (ANOVA, $p > 0.05$) hemolysis before aPDT procedure and after 30 min of incubation in the dark, since no significant differences were observed between LC and FORM, in NaCl solutions with percentages higher than 0.3% (**Figure 2.10a**). After 6 h of incubation in the dark, it was possible to observe 94% (ANOVA, $p < 0.05$) of hemolysis in 0.3% of NaCl solution in FORM, 17% higher than the hemolysis achieved in LC. However, in solutions with higher percentages of NaCl, no hemolysis was achieved. For FORM at 10 μM (**Figure 2.10b**), significant (ANOVA, $p < 0.05$) hemolysis was attained for 0.3% and 0.5% of NaCl solution before aPDT protocol (c.a 87% and 5.9%, respectively). However, after 6 h of incubation in the dark, 27% of hemolysis was observed in 0.5% of NaCl solution. No significant differences (ANOVA, $p > 0.05$) were observed between dark control and irradiated FORM.

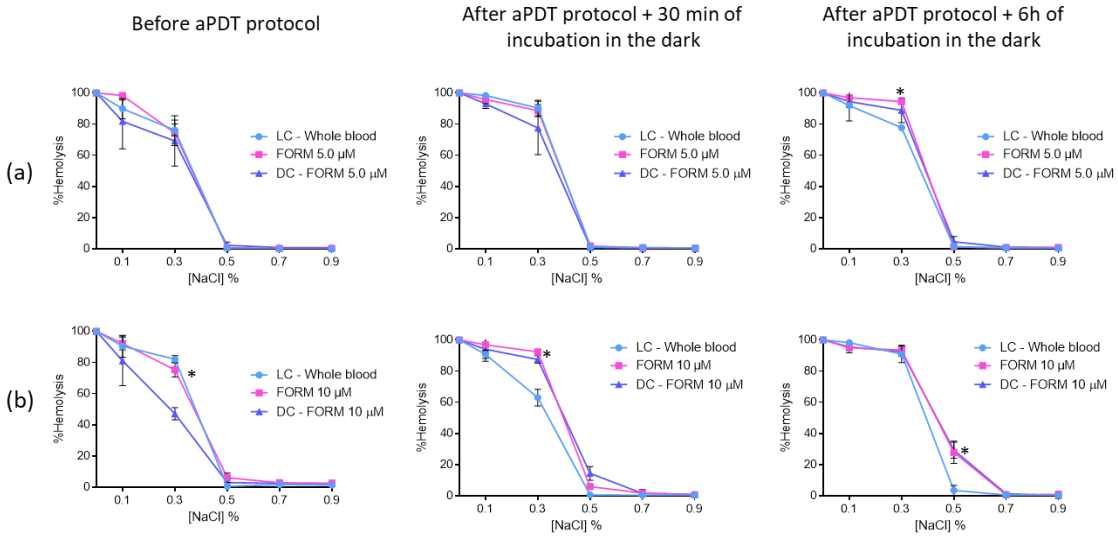


***Figure 2.10**: Erythrocyte osmotic fragility after the addition of the treated plasma with 5.0 (a) and 10 μM (b) of FORM to the concentrated erythrocytes before and after aPDT treatment under white light (380–700 nm) with an irradiance of 150 mW.cm$^{-2}$. Light control (plasma under light+concentrated erythrocytes) and dark control (plasma incubated with FORM without irradiation+concentrated erythrocytes) were included. Values represent the average of three independent experiments; error bars indicate the standard deviation. Lines just combine the experimental points. * $p < 0.05$ (relatively to the LC).*

When the concentrated erythrocytes was a mixture with plasma treated with Tri-Py(+)-Me at 5.0 μM (**Figure 2.11a**), it was observed that significant hemolysis in the 0.5% NaCl solution only occurred after the aPDT protocol: 15% after 30 min and 22% after 6 h of dark incubation. In the solutions with 0.7% and 0.9% of NaCl, no hemolysis was observed.

At the higher concentration (10 μM, **Figure 2.11b**) of Tri-Py(+)-Me, a similar profile was attained at 0.7% and 0.9% NaCl concentrations. For the 0.5% NaCl solution, significant hemolysis was observed before [39% (ANOVA, $p < 0.05$)] and after aPDT protocol, with dark incubations of 30 min [35% (ANOVA, $p < 0.0.5$)] and 6 h [74% (ANOVA, $p < 0.05$)]. Also in this case, no significant differences were observed between dark control and irradiated Tri-Py(+)-Me, with the exception of DC after 6 h of dark incubation. In this case, the hemolysis observed for irradiated Tri-Py(+)-Me was higher than the one observed for DC (75% vs 51% (ANOVA, $p < 0.05$)).

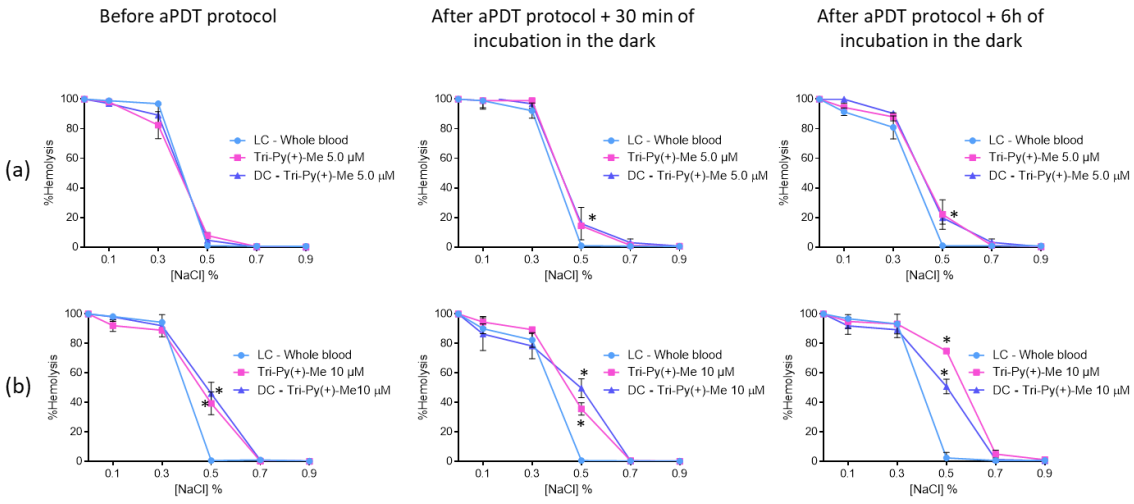


***Figure 2.11****: Erythrocyte osmotic fragility after the addition of the treated plasma with 5.0 (a) and 10 μM (b) of Tri-Py(+)-Me to the concentrated erythrocytes before and after aPDT treatment under white light (380-–700 nm) with an irradiance of 150 mW.cm$^{-2}$. Light control (plasma under light + concentrated erythrocytes) and dark control (plasma incubated with Tri-Py(+)-Me without irradiation + concentrated erythrocytes) were included. Values represent the average of three independent experiments; error bars indicate the standard deviation. Lines just combine the experimental points.*

The erythrocyte osmotic fragility results with MB revealed that this PS did not endorse hemolysis before and after aPDT for the two concentrations evaluated (**Figure 2.12**). The only exception observed was for 0.3% NaCl solution, where the hemolysis of MB at 5.0 μM [65% (ANOVA, $p < 0.05$)] and the respective DC (75% ($p < 0.05$)) was lower than the one observed for the LC (83% (ANOVA, $p < 0.05$)). Nevertheless, this feature

seems to be irrelevant after 6 h of incubation in the dark, since no differences were observed between LC, DC, and irradiated MB.

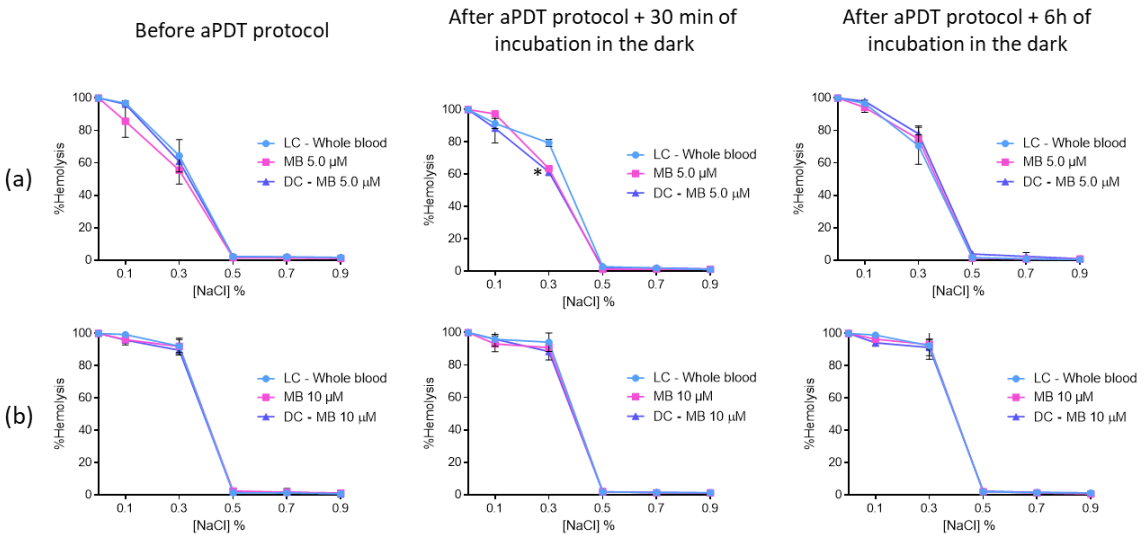


***Figure 2.12**: Erythrocyte osmotic fragility after the addition of the treated plasma with 5.0 (a) and 10 µM (b) of MB to the concentrated erythrocytes before and after aPDT treatment under white light (380–700 nm) with an irradiance of 150 mW·cm$^{-2}$. Light control (plasma under light+concentrated erythrocytes) and dark control (plasma incubated with MB without irradiation+concentrated erythrocytes) were included. Values represent the average of three independent experiments; error bars indicate the standard deviation. Lines just combine the experimental points. * $p < 0.05$ (relatively to the LC).*

## 5. Discussion

*C. albicans* is a commensal microorganism colonizing the gastrointestinal tract, skin, oral cavity, and reproductive tract in an asymptomatic and healthy way, but, under specific conditions, may cause nosocomial infections through the bloodstream [69]. Our results demonstrated that *C. albicans* is susceptible to aPDT in PBS, confirming the data in the literature that shows several examples where *C. albicans* planktonic cells as well their biofilm forms are efficiently photoinactivated in the presence of light and several PSs [54,70–72]. The results attained with FORM in PBS when compared to its constituents, Tri-Py(+)-Me and Tetra-Py(+)-Me (**Figure 2.4**), encouraged further use to extend its benefits in the inactivation of *C. albicans* in blood plasma and/or whole blood. The results achieved with Tri-Py(+)-Me in PBS were also significant, since after 60 min of irradiation a decrease of 3.2 $\log_{10}$ ($p < 0.05$) in the fungus survival was achieved and after 270 min the detection limit was reached. As far as we are aware, this was the first time that this porphyrin derivative

was used in the inactivation of *C. albicans* in plasma. Since Tetra-Py(+)-Me was the less efficient porphyrinic PS in the inactivation of *C. albicans* in PBS, it was not included in the following studies.

It is remarkable the efficiency of the porphyrinic PSs in the photoinactivation of *C. albicans* when compared to the efficiency of the reference MB, already approved for disinfection of blood plasma. In fact, MB was reported as efficient PS in the inactivation of this fungus, however, this was in concentrations 10× higher than those used in this study [72,73]. Moreover, the efficiency of MB in the inactivation of the fungus is highly dependent on the pH of the solution and the phototoxic effects only occur in the presence of saline solutions (non-buffered medium) [72].

The PBS studies give important information on the photoinactivation profile and effectiveness of the PSs, but considering the technological extension of the aPDT approach, it is also important to evaluate the efficiency of the PSs in different matrices and to compare the results with the ones obtained in buffer solutions. In this study, the evaluation of the efficiency of FORM, Tri-Py(+)-Me and MB in blood plasma and whole blood was crucial to assess the possible translation to the clinic environment. Keeping in mind that blood elements, such as erythrocytes, must not be affected by the aPDT treatment approach, the erythrocyte osmotic fragility was assessed in order to choose the safe PSs concentrations (concentrations that did not promoted hemolysis). As was observed for Tri-Py(+)-Me [5], none of the PSs had promoted hemolysis at 5.0 and 10 μM at the isotonic conditions [74] (the nonstress condition (0.9% NaCl)), which confers safety for their potential used in disinfection in whole blood and /or plasma. Although hemolysis tends to increase with the presence of reactive oxygen species (ROS) resulting from PS activation [5], this was not observed in the conditions studied, since no significant differences were observed before and after the aPDT treatment. Moreover, the fact that similar the results were attained in the dark controls and after PS irradiation, which led us to conclude that the irradiation protocol did not induce hemolysis.

At 5.0 and 10 μM concentrations of FORM, as well for Tri-Py(+)-Me, hemolysis was observed for the lowest NaCl concentrations (**Figure 2.5**). These lower NaCl concentration solutions are stress-inducing solutions, since the salt concentration in the extracellular medium is lower (hypotonic condition) than within the cell (hypertonic condition). The water

enters the cell by osmosis, causing its lysis. The same hemolysis profile was achieved for the light controls (LC-Whole blood), showing, once again, that the aPDT protocol is not responsible for the red cells hemolysis. Comparing the results of porphyrinic PSs with the ones achieved for MB, were no hemolysis was observed for 5.0 μM for the 0.5, 0.7 and 0.9% of NaCl solution and for the highest concentrations of the PSs hemolysis was only achieved for the 0.3% NaCl solution (**Figure 2.6**), led us to consider that DMSO used to dissolve the porphyrinic PS has an important contribution for the hemolysis profile observed in FORM and Tri-Py(+)-Me. In fact, when the erythrocyte osmotic fragility was evaluated with 1%, 2%, and 4% of DMSO (**Figure 2.7**) the profile of hemolysis was similar to ones achieved for FORM and Tri-Py(+)-Me at 5.0, 10, and 20 μM; thus lower percentages of DMSO (1% and 2%) did not promote significant hemolysis in the 0.7% and 0.9% NaCl solutions before and after the aPDT protocol. However, in the solutions with lower percentages of NaCl, the hemolysis rates increased. For example, comparing the case of erythrocyte osmotic fragility results obtained with FORM at 5.0 μM with the ones attained with 1% of DMSO in the 0.5% NaCl solution, it was possible to observe that after the aPDT protocol, FORM induced 38% of hemolysis while DMSO endorsed 14%, which means that in fact, FORM only promoted 24% of hemolysis. This is more evident after the aPDT protocol, when FORM promoted 51% of hemolysis and DMSO endorsed 40%. DMSO is widely used in in vitro assays as a solvent of antibacterial agents and its ability to cross cell membranes is known to have an important biological feature. Regardless, with the percentages used in this work, DMSO has no effects in the survival of pathogens, including *C. albicans*. However, the hemolysis promoted by this solvent may cause some controversy regarding the use of DMSO as a solvent of PSs for blood plasma and whole blood disinfection mediated by aPDT. In this case, the choice of other drug-delivery systems such as micelles, liposomes, or the immobilization of the PS in a support may be a more secure option [73]. Nevertheless, it is important to emphasize, as already mentioned, that neither FORM or Tri-Py(+)-Me promoted hemolysis at 5.0 and 10 μM under the isotonic conditions studied.

The safe concentrations obtained for FORM and Tri-Py(+)-Me were used to photoinactivate *C. albicans* in blood plasma and whole blood. These two PSs efficiently inactivate this fungus in blood plasma, promoting a decrease in the survival of the fungus higher than the reference MB (**Figure 2.8**). In this case, Tri-Py(+)-Me seems to be the more efficient PS on the inactivation of *C. albicans* in blood plasma, since the decrease attained

in the survival of the fungus after 270 min of irradiation was higher than the one observed for FORM (2.5 $\log_{10}$ *vs* 1.7 $\log_{10}$ for 10 μM of each PS). This was quite surprising, since in the PBS assays FORM was the most efficient PS in photoinactivation of *C. albicans*. This may be explained, not only by the complexity of the plasma matrix, which is rich in several proteins that can interfere by trapping the PS, but also due to the complexity of FORM. This formulation is constituted by 5 cationic porphyrins with different number of charges. The constituents of this formulation can, in a complex environment such as plasma, lose their ability to efficiently bind to the microbial membrane cells, decreasing their photodynamic efficiency. In regards to the reference MB, this was the less efficient PS in the inactivation of the fungus (promoting a decrease of *c.a* 0.5 $\log_{10}$ for the higher tested concentration), maintaining the inactivation profile at a constant level between 90 and 270 min. The reduced efficiency of MB can be explained by the fact that this PS has only a positive charge. It is well known that cationic PS with 3 or 4 charges are more effective for photoinactivating microorganisms, namely Gram-negative bacteria and fungi, than neutral PS or PS with only one or two positive charges [46,74]. The observed photodegradation of MB can also, at least in part, justify the poor ability of these PS in the photoinactivation of *C. albicans* in blood plasma. Moreover, MB at 5.0 and 10 μM was not more efficient than FORM and Tri-Py(+)-Me at the same concentrations, so it is expected that, at the same amounts used in the approved methodology for plasma disinfection mediated by light and MB (where the PS is used at 0.8–1.2 μM), the porphyrinic PSs continued to be more efficient than MB [75].

While it is known that the PSs approved to disinfect plasma are not approved for disinfecting concentrated erythrocytes due to the negative effects observed after aPDT, the ability of FORM and Tri-Py(+)-Me to photoinactivate *C. albicans* in whole blood was still studied (**Figure 2.9**). However, under the aPDT protocol selected, the decrease of the fungus survival attained 0.7 $\log_{10}$ for the higher concentrations of each PSs. Once again, the complexity of the blood matrix may be the answer for this limited efficiency of the PSs. The nonspecific binding of the PS to blood proteins and to the high number of elements coating cell membranes can decrease the efficiency of the photoinactivation [5]. Moreover, while the microorganisms are in the suspension in plasma, in the whole blood pathogens may be in suspension or associated with cells (intracellular or extracellular), which can undermine the interaction of the ROS with the membrane of the cells [7]. Nevertheless the potential

application of FORM and Tri-Py(+)-Me in the photoinactivation of *C. albicans* in blood plasma is very promising.

In order to investigate whether treated plasma mediated by aPDT could damage the erythrocyte membranes after the transfusion, the erythrocyte osmotic fragility after aPDT of plasma with FORM, Tri-Py(+)-Me at concentrations of 5.0 and 10 μM was studied. The results were also compared to the ones achieved for MB at the same concentrations. This study aims to simulate plasma transfusions and to evaluate the possible erythrocyte damage that aPDT-treated plasma transfusion can cause in the receptor. None of the PSs had promoted hemolysis at 5.0 and 10 μM at the isotonic conditions (0.9% NaCl) before aPDT and after aPDT, followed by dark incubations of 30 min and 6 h. These results confirm the safety of the use of FORM and Tri-Py(+)-Me in the disinfection of plasma.

## 6. Conclusions

It is obvious that there is a lack of efficient methods for the inactivation of pathogens in blood plasma and whole blood [5,7,18], and it is crucial to develop new strategies to inactivate microorganism in plasma and/or blood [10]. aPDT can represent an alternative to the conventional disinfection techniques since it is a non-antibiotic approach that has been shown to be effective in the photoinactivation of several pathogens. One of the disinfection techniques approved for pathogenic inactivation in plasma considered the use of MB as PS in an aPDT approach. This work shows that the porphyrinic formulation (FORM) and the Tri-Py(+)-Me are promising PSs in the inactivation of *C. albicans* in blood plasma, causing higher inactivation rates than MB. Moreover, these porphyrinic PSs had shown no significant negative effects on the erythrocytes in isotonic conditions when hemolysis was evaluated in whole blood and after the addition of treated plasma to the concentrated blood cells. However, further studies are needed to overcome the barriers that the complex matrix of whole blood promotes in order to improve the efficacy of FORM and Tri-Py(+)-Me in the photoinactivation of *C. albicans* in whole blood.

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# Chapter III- General Conclusions and Future Perspectives

## 1. General conclusions

There is a lack of efficient methods for the inactivation of pathogens in whole blood and blood products. At this time, the method commonly used for disinfecting plasma and protein concentrates combines the use of an organic solvent and a detergent, which should be removed after treatment because they may induce negative effects on blood cells and plasma proteins. This work reports the use of a porphyrin with three positive charges (Tri-Py(+)-Me) and a PS formulation (FORM) in the photoinactivation of *C. albicans* in plasma and whole blood. *C. albicans* is an opportunistic fungus responsible for dangerous systemic infections in immunocompromised individuals, mainly cancer patients and HIV infected patients. The main conclusions are summarized below:

- *C. albicans* was effectively inactivated by aPDT in PBS, being the FORM the most efficient of the tested PS, showing the highest photoinactivation rate in a shortest light exposure time.

- Tri-Py(+)-Me and FORM, at 5.0 and 10 μM, showed no significant negative effects on erythrocytes in isotonic conditions (0.9% NaCl), but the concentration of 20 μM cause high hemolysis rates at 0.9% NaCl. Besides, MB at all tested concentrations (5.0, 10 and 20 μM) did not show any significant hemolysis.

- DMSO used to prepare stock solutions of FORM and Tri-Py(+)-Me, at 1 and 2% did not promote hemolysis in isotonic conditions, however at 4% this solvent showed high hemolysis rates at 0.9% NaCl, being not indicated as a solvent of PSs for blood disinfection.

- The porphyrinic formulation FORM and the Tri-Py(+)-Me are promising PSs in the inactivation of *C. albicans* in plasma, causing higher inactivation rates than MB. Although MB is currently approved for disinfection of plasma, it was the less effective PS in *C. albicans* photoinactivation.

- Despite the promising results obtained in plasma by the porphyrinic PSs, in the whole blood a reduction of only 0.7 $\log_{10}$ was achieved, which can be due to the complexity of the blood matrix.

- aPDT-treated plasma with FORM and Tri-Py(+)-Me seems to be safe once none of the PSs had promoted hemolysis at 5.0 and 10 μM at the isotonic conditions (0.9% NaCl) before aPDT (untreated plasma) and after aPDT (treated plasma), both in short (30 min) and long (6 h) term evaluation.

## 2. Future perspectives

Although DMSO has no effects on the survival of *C. albicans*, the hemolysis caused by this solvent at higher porphyrin concentrations requires alternatives approaches for the delivery of the PS. For this reason, the choice of other drug-delivery systems such as micelles, liposomes, or immobilization of the PS in a support may be a more secure option. Additionally, further studies are needed to overcome the barriers that the complex matrix of whole blood promotes, like the nonspecific binding of the PS to blood proteins and to the high number of elements coating cell membranes that can decrease the efficiency of the photoinactivation. It would be interesting to perform uptake assays to help to understand how the proteins and cells in blood matrix may influence the interaction of PS with the microorganism and then take this into account to improve the inactivation rates.

# New nitroindazole porphyrin conjugates: synthesis, characterization and antibacterial properties

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**Keywords:** Porphyrin; Knoevenagel condensation; Indazole; Photosensitizer; Antimicrobial Photodynamic Therapy (aPDT); MRSA, Potassium iodide

# Appendix

## 1. Abstract

The synthesis of a new porphyrin-indazole hybrids by a Knoevenagel condensation of 2-formyl-tetraphenylporphyrin and *N*-methyl-nitroindazole derivatives is reported. The target compounds were isolated in moderate to good yields (32-57%) and some of the isolated porphyrin-indazole conjugates showed a good performance in the generation of singlet oxygen when irradiated with light. Their efficiency as photosensitizers in the photoinactivation of methicillin resistant *Staphylococcus aureus*–MRSA was evaluated. All derivatives showed to be able to photoinactivate the MRSA bacterium. Despite being the least efficient in singlet oxygen generation, compound **3a** appears to be the most promising PS in the photoinactivation of this bacterium. The addition of potassium iodide significantly potentiated the aPDT process mediated by all the porphyrin-indazole conjugates analysed. In fact, the combined action of antimicrobial properties of nitroindazole porphyrins enhanced by KI action appear to be promising in the photoinactivation of *S. aureus* MRSA.

## 2. Introduction

Porphyrins and indazoles derivatives are *N*-heterocycles with recognized relevance under different contexts. Porphyrins are well-known by their role in vital processes like respiration, photosynthesis, electron transportation and storage of relevant molecules [1,2]. Additionally, these natural macrocycles but also the synthetic analogues display photophysical and photochemical features particularly attractive to be used in a wide range of fields like supramolecular chemistry, catalysis, electronic materials, sensors and medicine [3]. A relevant application of porphyrins in medicine is related with their role as photosensitizers in Photodynamic therapy (PDT) of tumours and more recently in the photodynamic inactivation of microorganisms (aPDT). The principles behind both treatments are the same and require the excitation of photosensitizer (PS) by light in the presence of molecular oxygen to produce reactive oxygen species (ROS), namely singlet oxygen ($^1O_2$) responsible by the destruction of the cancer cells or the inactivation of microorganisms [4-7]. These treatments have attracted great attention as an emerging clinical tool and as alternatives to traditional chemotherapy or antibiotic therapy. Since the photodynamic process is a multitarget approach and occurs only in the irradiated region, the possibility of photoresistance development is unlikely [5, 8-10].

Indazole derivatives although with a limited occurrence in nature are also being successfully explored in the design of molecules with adequate features to be used for instance as anti-tumoral, antimicrobial and anti-inflammatory drugs [11-13]. It is known that the functionalization of the porphyrin core with moieties with relevant biological features can afford porphyrins with an improvement biological performance [9, 14]. In this context, 2-formyl-5,10,15,20-tetraphenylporphyrin **1** has revealed to be a versatile template for the porphyrinic core modification [15]. In fact, β-formyl porphyrins can be easily manipulated by recurring to different approaches namely those based on McMurry, Schiff's base, Horner-Emmons, Grignard, Wittig, cycloaddition and Knoevenagel reactions [16].

Under the context of the Knoevenagel condensation, Pornomarev and co-workers reported the first condensations involving the Cu(II) complex of 2-formyl-porphyrin **1** with malonic acid and its methyl or ethyl esters affording the corresponding porphyrin derivatives in yields higher than 87%[17]. A decade later, Chen and co-workers explored this approach to couple a series of formyl derivatives bearing olefin bridges of varying lengths in the β-pyrrolic position of the Ni(II) complex of *meso*-tetrakis(4-isopropylphenyl)porphyrin, with diethyl malonate, ethyl cyanoacetate, malononitrile, and *N*,*N'*-diethylthiobarbituric acid in the presence of aluminium oxide [18, 19].

More recently, Mandeep, Sankar and co-workers used the condensation of malonitrile or cyanoacetic with a series of β-formyl *meso*-tetraarylporphyrins as free-bases or coordinated with different metals (*e.g.* Ni(II), Cu(II)) to afford the expected products in excellent yields. In some of their studies it was evaluated the potential of this type of derivatives to be used as colorimetric chemosensors for $CN^-$ and $F^-$ detection [20-22].

The Knoevenagel condensation between the Zn(II) complex of 2-formyl porphyrinic derivatives and a series of adequate methylene active compounds is also being successfully explored by different researchers groups in the development of sensitizers for Dye-Sensitized Solar Cells (DSSC). Attractive power conversion efficiencies ranging from 5 to 8% have been reported [23-32].

In 2017, Officer *et al.* reported that the Knoevenagel condensation between 2-formyl porphyrin **1** and a series of *para* substituted phenylacetonitriles afforded the expected porphyrinylacetonitriles. The authors mentioned that the reactions with phenylacetonitriles bearing strong electron-donating groups need to be mediated with strong bases, such as DBU

or KO*t*Bu. The authors explore this approach to prepare symmetrical and unsymmetrical porphyrinic dyads by using 1,4-phenylenediacetonitrile, in the presence of DBU or sodium methoxide [33].

Recently, we reported an efficient synthetic access to a series of nitroindazolylacetonitriles from the reaction of adequate *N*-methyl-nitroindazole with 4-chlorophenoxyacetonitrile[34] (*vide* infra **Scheme 1**). Considering the potential of these compounds to act as active methylene components and the reactivity of 2-formylTPP (**1**) in Knoevenagel condensations, we envisaged that this approach could allow the access to porphyrinic derivatives decorated with nitroindazole units.

So, herein we report the synthetic access to new porphyrin-indazole hybrids **3a-e** by recurring to the Knoevenagel reaction between the *N*-methyl-nitroindazolylacetonitriles **2a-e** and the 2-formyl-5,10,15,20-tetraphenylporphyrin, **1** (**Scheme 1**). The efficacy of these derivatives to generate oxygen singlet prompt us to evaluate also their photodynamic efficacy against a MRSA *Staphylococcus aureus* bacterium. *S. aureus* is a gram-positive bacterium which in normal conditions is commensal in humans and animals, but can easily become pathogenic, causing frequently skin, respiratory, bone, soft tissue and endovascular infections [35,36]. As *S. aureus* often develops resistance to multiple antibiotics, namely to β-lactam antibiotics such as methicillin (MRSA strains) [37], this bacterium is one of the major causes of health care associated and community associated infections[38]. Although aPDT is considered more effective against Gram-positive bacteria than against Gram-negative bacteria [39], aPDT experiments with potassium iodide (KI), a well-known potentiator of aPDT [40-46] , were included in order to increase the effectiveness of the new porphyrin-indazole hybrids **3a-e.**

## 3. Experimental section

### 3.1. General remarks

Melting points were measured using a Buchi Melting Point B-540 apparatus. Electrospray ionization mass spectra (ESI) were acquired with a Micromass Q-Tof 2 (Micromass, Manchester, UK), operating in the positive ion mode, equipped with a Z-spray source, an electrospray probe and a syringe pump. Source and desolvation temperatures were 80 ºC and 150 ºC, respectively. Capillary voltage was 3000 V. The spectra were acquired at a nominal resolution of 9000 and at cone voltages of 30 V. Nebulisation and collision gases

were $N_2$ and Ar, respectively. Compound solutions in methanol were introduced at a 10 μL $min^{-1}$ flow rate. $^1H$ and $^{13}C$ solution NMR spectra were recorded on Bruker Avance 500 (500 and 125 MHz, respectively) spectrometer. $CDCl_3$ was used as solvent and tetramethylsilane (TMS) as the internal reference; the chemical shifts are expressed in δ (ppm) and the coupling constants (*J*) in Hertz (Hz). Unequivocal $^1H$ assignments were made using 2D COSY ($^1H/^1H$), while $^{13}C$ assignments were made on the basis of 2D HSQC ($^1H/^{13}C$) and HMBC (delay for long-range *J* C/H couplings were optimized for 7 Hz) experiments. Elemental analyses were performed on a LECO CHNS-932 apparatus. Column chromatography was carried out using silica gel (Merck, 35-70 mesh). Analytical TLC was carried out on precoated sheets with silica gel (Merck 60, 0.2 mm thick).

All chemicals were used as supplied. Solvents were purified or dried according to the literature procedures [47]. The 2-formyl-5,10,15,20-tetraphenylporphyrin **1** was prepared from 5,10,15,20-tetraphenylporphyrinatocopper(II), *N*,*N*-dimethylformamide (DMF) and phosphorus oxychloride ($POCl_3$), according to literature procedure [48]. *N*-methyl-nitroindazoles **2a-e** were prepared by *N*-methylation of the appropriate nitro-1*H*-indazoles in the presence of NaOH, followed by reaction with 4-chlorophenoxyacetonitrile.as reported in literature [34].

### 3.2. Synthesis

**Knoevenagel reaction. General procedure:** To a solution of 2-formyl-5,10,15,20-tetraphenylporphyrin **1** (20 mg, 31.2 mmol) in THF (5 mL) was added the appropriate *N*-methyl-nitroindazole **2a-e** (2 equiv., 62.4 mmol, 134.7 mg) and an excess of piperidine (0.5 mL). The mixture was stirred and heated under reflux for 36 h. After cooling the solvent was removed under reduced pressure and the crude mixture was purified by column chromatography (silica gel) using toluene as the eluent. The compounds isolated were then crystallized from $CH_2Cl_2$-hexane and fully characterized by NMR, mass and UV-Vis techniques. The yields are summarized in Scheme 1.

**(*Z*)-2-(2-(2-methyl-4-nitro-1*H*-indazol-5-yl)-acrilonitril-3-yl-5,10,15,20-tetraphenylporphyrin, 3a.$^1$H NMR (500 MHz, $CDCl_3$)**: *δ* 9.61 (1H, s, H-3), 8.98 (1H, d, *J* = 4.8 Hz, H-β), 8.90 (1H,d, *J* = 4.8 Hz, H- β), 8.82 (1H, d, *J* = 4.8 Hz, H-β), 8.79 and 8.78 (2H, AB System, *J* = 4.8 Hz, H-β), 8.72 (1H, d, *J* = 4.8 Hz, H-β), 8.57 (1H, s, H-3´´), 8.34-8.32 (2H, m, H-*o*-Ph), 8.23-8.19 (4H, m, H-*o*-Ph), 8.15 (2H, d, *J* = 7.6 Hz, H-*o*-Ph), 8.01

(1H, d, *J* = 8.7 Hz, H-7´´), 7.87-7.73 (9H, m, H-*m*,*p*-Ph), 7.60 (2H, t, *J* = 7.6 Hz, H-*m*-Ph), 7.49 (1H, t, *J* = 7.6 Hz, H-*p*-Ph), 7.06 (1H, s, H-1´), 7.01 (1H, d, *J* = 8.7 Hz, H-6´´), 4.37 (3H, s, -$CH_3$), -2.61 (2H, s, N-*H*) ppm. **$^{13}$C NMR (125 MHz, $CDCl_3$)**: $\delta$ 149.6, 142.4, 142.2, 142.0, 141.7, 141.6, 138.7, 135.3, 134.63, 134.57, 134.4, 133.2-129.7 (C-β), 128.8, 128.21, 128.16, 127.91, 127.88, 127.85, 127.4, 127.0, 126.9, 126.8, 126.3, 124.4, 121.8, 120.6, 120.4, 119.3, 116. 9, 116.2, 109.5, 41.1 (-$CH_3$) ppm. **MS-ESI(+):** *m/z* 841.3 $[M+H]^+$. **UV-Vis** (DMF): $\lambda_{max}$ (log ε) 429 (4.70), 524 (3.59), 561 (3.20), 601 (3.07), 658 (3.09) nm.

**(*Z*)-2-(2-(1-methyl-5-nitro-1*H*-indazol-4-yl)-acrilonitril-3-yl-5,10,15,20-tetraphenylporphyrin, 3b.$^{1}$H NMR (500 MHz, $CDCl_3$)**: $\delta$ 9.68 (1H, s, H-3), 8.99 (1H, d, *J* = 4.8 Hz, H- β), 8.91 (1H,d, *J* = 4.8 Hz, H- β), 8.82 (1H, d, *J* = 4.8 Hz, H-β), 8.79-8.75 (3H, m, H-β), 8.36-8.34 (2H, m, H-*o*-Ph), 8.30 (1H, d, *J* = 9.3 Hz, H-6´´), 8.24-8.19 (4H, m, H-*o*-Ph), 8.08 (1H, s, H-3´´), 8.04 (2H, d, *J* = 7.4 Hz, H-*o*-Ph), 7.86-7.73 (9H, m, H-*m*,*p*-Ph), 7.52 (1H, d, *J* = 9.3 Hz, H-7´´), 7.34 (2H, t, *J* = 7.4 Hz, H-*m*-Ph), 7.15 (1H, t, *J* = 7.4 Hz, H-*p*-Ph), 6.84 (1H, s, H-1´), 4.19 (3H, s, -$CH_3$), -2.61 (2H, s, N-*H*) ppm. **$^{13}$C NMR (125 MHz, $CDCl_3$)**: $\delta$ 143.5, 141.9, 141.8, 141.72, 141.66, 140.8, 140.6, 135.3, 135.1, 134.63, 134.57, 134.2, 133.5-129.5 (C-β), 129.1, 128.2, 127.90, 127.86, 127.7, 127.2, 127.0, 126.9, 126.8, 126.4, 124.1, 123.2, 121.8, 120.6, 120.4, 119.5, 116.7, 109.7, 104.9, 36.2 (-$CH_3$) ppm. **MS-ESI(+):** *m/z* 841.3 $[M+H]^+$. **UV-Vis** (DMF): $\lambda_{max}$ (log ε) 430 (5.18), 523 (4.16), 560 (3.67), 602 (3.64), 658 (3.56) nm.

**(*Z*)-2-(2-(2-methyl-5-nitro-1*H*-indazol-4-yl)-acrilonitril-3-yl-5,10,15,20-tetraphenylporphyrin, 3c.$^{1}$H NMR (500 MHz, $CDCl_3$):** $\delta$ 9.66 (1H, s, H-3), 8.99 (1H, d, *J* = 4.8 Hz, H- β), 8.91 (1H,d, *J* = 4.8 Hz, H- β), 8.83 (1H, d, *J* = 4.8 Hz, H-β), 8.80-8.74 (3H, m, H-β), 8.35-8.34 (2H, m, H-*o*-Ph), 8.23-8.18 (4H, m, H-*o*-Ph), 8.13-8.11 (2H, m, H-3'' and H-6´´), 8.02 (2H, d, *J* = 7.4 Hz, H-*o*-Ph), 7.86-7.73 (10H, m, H-*m*,*p*-Ph and H-7´´), 7.34 (2H, t, *J* = 7.4 Hz, H-*m*-Ph), 7.21 (1H, t, *J* = 7.4 Hz, H-*p*-Ph), 6.80 (1H, s, H-1´), 4.24 (3H, s, -$CH_3$), -2.62 (2H, s, N-*H*) ppm. **$^{13}$C NMR (125 MHz, $CDCl_3$):** $\delta$ 149.2, 144.0, 141.0, 141.72, 141.70, 141.68, 141.2, 135.3, 134.62, 134.56, 134.2-129.5 (C-β), 128.2, 127.93, 127.89, 127.8, 127.7, 127.2, 127.0, 126.9, 126.8, 126.1, 122.2, 122.0, 121.7, 120.6, 120.5, 119.5, 118.9, 116.8, 105.6, 41.2 ppm. **MS-ESI(+):** *m/z* 841.3 $[M+H]^+$. **UV-Vis** (DMF): $\lambda_{max}$ (log ε) 430 (5.03), 523 (4.02), 560 (3.56), 602 (3.52), 658 (3.42) nm.

**(*Z*)-2-(2-(2-methyl-6-nitro-1*H*-indazol-4-yl)-acrilonitril-3-yl-5,10,15,20-tetraphenylporphyrin, 3d.$^{1}$H NMR (500 MHz, $CDCl_3$):** $\delta$ 9.72 (1H, s, H-3), 8.97 (1H, d, *J* = 4.8 Hz, H- β), 8.89 (1H,d, *J* = 4.8 Hz, H- β), 8.81-8.75 (4H, m, H-β), 8.35-8.33 (2H, m, H-*o*-Ph), 8.24-8.19 (4H, m, H-*o*-Ph), 8.06-8.04 (3H, m, H-*o*-Ph and H-3´´), 7.84-7.72 (11H, m, H-*m*,*p*-Ph, H-4´´ and H-5´´), 7.30 (2H, t, *J* = 7.6 Hz, H-*m*-Ph), 7.12 (1H, t, *J* = 7.6 Hz, H-*p*-Ph), 7.02 (1H, s, H-1´), 4.22 (3H, s, -$CH_3$), -2.62 (2H, s, N-*H*) ppm. **$^{13}$C NMR (125 MHz, $CDCl_3$):** $\delta$ 146.5, 144.7, 144.6, 142.0, 141.8, 141.77, 141.75, 135.4, 134.62, 134.56, 134.3, 133.2-129.6 (C-β), 128.1, 127.9, 127.8, 127.5, 127.0, 126.84, 126.75, 124.8, 124.1, 121.9, 121.7, 121.6, 120.4, 120.3, 119.7, 117.4, 116.9, 116.9, 41.3 ppm. **MS-ESI(+):** *m/z* 841.3 $[M+H]^+$. **UV-Vis** (DMF): $\lambda_{max}$ (log ε) 430 (5.24), 523 (4.34), 562 (3.83), 601 (3.79), 658 (3.73) nm.

**(*Z*)-2-(2-(1-methyl-7-nitro-1*H*-indazol-4-yl)-acrilonitril-3-yl-5,10,15,20-tetraphenylporphyrin, 3e.$^{1}$H NMR (500 MHz, $CDCl_3$):** $\delta$ 9.68 (1H, s, H-3), 8.99 (1H, d, *J* = 4.9 Hz, H-β), 8.91 (1H, d, *J* = 4.9 Hz, H-β), 8.82 (1H, d, *J* = 4.9 Hz, H-β), 8.79-8.74 (4H, m, H-β), 8.37-8.35 (2H, m, H-*o*-Ph), 8.29 (1H, d, *J* = 9.3 Hz, H-6´´), 8.23-8.18 (4H, m, H-*o*-Ph), 8.07-8.03 (3H, m, H-*o*-Ph and H-3´´), 7.84-7.72 (9H, m, H-*m*,*p*-Ph), 7.51 (1H, d, *J* = 9.3 Hz, H-5´´), 7.34 (2H, t, *J* = 7.7 Hz, H-*m*-Ph), 7.18-7.13 (1H, m, H-*p*-Ph), 6.84 (1H, s, H-1´), 4.17 (3H, s, -$CH_3$), -2.61 (2H, s, N-*H*) ppm. **$^{13}$C NMR (125 MHz, $CDCl_3$):** $\delta$ 143.5, 141.9, 141.8, 141.72, 141.67, 140.8, 140.6, 137.9, 135.3, 135.0, 134.64, 134.58, 134.2, 133.4-129.52 (C-β), 129.1, 128.3, 128.2, 127.91, 127.87, 127.7, 127.2, 127.0, 126.9, 126.8, 126.4, 125.3, 124.5, 124.1, 124.0, 123.2, 121.8, 120.6, 120.4, 119.5, 119.1, 116.7, 109.7, 104.9, 36.2 ppm. **MS-ESI(+):** *m/z* 841.3 $[M+H]^+$. **UV-Vis** (DMF): $\lambda_{max}$ (log ε) 431 (5.33), 523 (4.16), 562 (3.82), 602 (3.80), 659 (3.74) nm.

### 3.3. Spectrophotometric and Spectrofluorometric Measurements

The absorption spectra were recorded on a UV-2501PC Shimadzu spectrophotometer and the fluorescence emission spectra were recorded on a Horiba Jobin-Yvon Fluoromax 3 spectrofluorimeter using DMF as solvent. The linearity of the fluorescence emission versus the concentration was checked in the concentration range used ($10^{-4}$-$10^{-6}$ M). The correction of the absorbed light was performed when it was necessary. The studied solutions were prepared by appropriate dilution of the stock solutions up to $10^{-5}$-$10^{-6}$ M. All the measurements were performed at 298 K.

Fluorescence quantum yields of all porphyrin-nitroindazole derivatives **3a-e** were measured using a solution of 5,10,15,20-tetraphnylporphyrin (**TPP**) in dimethylformamide (DMF) as standard ($\Phi_{Flu}$ = 0.11) [49].

### 3.4. Singlet oxygen generation

Solutions of porphyrin-nitroindazole derivatives **3a-e** in DMF ($Abs_{430} \approx 0.50$) were aerobic irradiated in quartz cuvettes with monochromatic light ($\lambda$ = 518 nm) in the presence of 9,10-dimethylanthracene (DMA, 30 μM). **TPP** was used as reference ($\Phi_{\Delta}$ = 0.65) [50]. The kinetics of DMA photooxidation was studied by following the decrease in its absorbance at 378 nm and the result registered in a first-order plot for the photooxidation of DMA photosensitized by derivatives **3a-e**, and **TPP** in DMF. The kinetics of DMA photooxidation in the absence of any compound was also studied and no significant photodegradation of DMA was observed under the same irradiation conditions. The results are expressed as mean and standard deviation obtained from three independent experiments.

### 3.5. Detection of Iodine Formation

In a 96 wells microplate, appropriate volumes of each compound at 5.0 μM and combinations of each compound at 5.0 μM and KI at 100 mM were incubated in the dark for 15 min and then irradiated with white light. The generation of iodine ($I_2$) was monitored by reading the absorbance at 340 nm at different pre-defined irradiation times. Controls of PBS and KI were also performed.

### 3.6. KI solution

Potassium iodide was provided by Biochem Chemopharma and the solutions of KI were prepared at 5M in sterile PBS and tested at 100mM in photodynamic assays. These solutions were prepared immediately before each assay.

### 3.7. Light sources

In aPDT assays were used an LED PROJECTOR (type: VEGA20, 20W, ~230V, 50/60 Hz) with an irradiance of 50 $mW.cm^{-2}$. The irradiances were measured with a Power Meter Coherent FieldMax-II Top combined with a Coherent PowerSens PS19Q energy sensor.

### 3.8. Characterization of microbial strains and microbial grown conditions

In this study was used a gram-positive bacterium *Staphylococcus aureus* DSM 25693, a methicillin-resistant (MRSA) strain that produces the staphylococcal enterotoxins S, E, A, C, H, G, and I. This gram-positive bacterium was isolated from a biological low respiratory tract sample of a hospitalized individuals [51].

The bacterium was maintained on Tryptic Soy Agar (TSA, Liofilchem) at 4 °C. Before each assay, a colony was transferred to 30 mL of Tryptic Soy Both (TSB, Liofilchem) and incubated for 18-24 h at 37 °C with constant stirring (120 rpm). Then 300 μL aliquots were transferred to new 30 mL TSB and incubated at the previous growth conditions, in order to reach the stationary phase, corresponding to a concentration of $10^8$-$10^9$ colony forming units per mL ($CFU.mL^{-1}$).

### 3.9. Photodynamic assays

After reaching the stationary phase the bacterial culture was diluted (1:10) in PBS, and the suspension was then distributed to the wells of a 12-well plate. Then, each PS (**3a-e**) solution previous sonicated for 15 min were added to the samples (5.0 μM of PS without KI) and dark controls (DC, 5.0 μM of PS without KI and in the absence of light). Similar photodynamic assays were performed with the combined addition of 5.0 μM of PS and 100 mM of KI, light (**LC**) and KI controls (**LC+KI**) were also performed. To promote the interaction of the PS with the bacterium, samples and controls were incubated before irradiation for 1 h with constant agitation in the dark. Then, samples with and without KI, light control (LC) and KI control (LC+KI) were irradiated under the conditions described above with constant agitation, while the dark controls were protected from light during the treatment. Photoinactivation efficiency of each PS was evaluated by quantifying the number of colony forming units (CFU) per volume ($CFU.mL^{-1}$). Aliquots of samples and each controls were taken at time 0 min (after incubation time) and at different irradiation times (30, 45, 60, 90 and 150 min). After this, serial dilutions were made and finally plated in TSA.

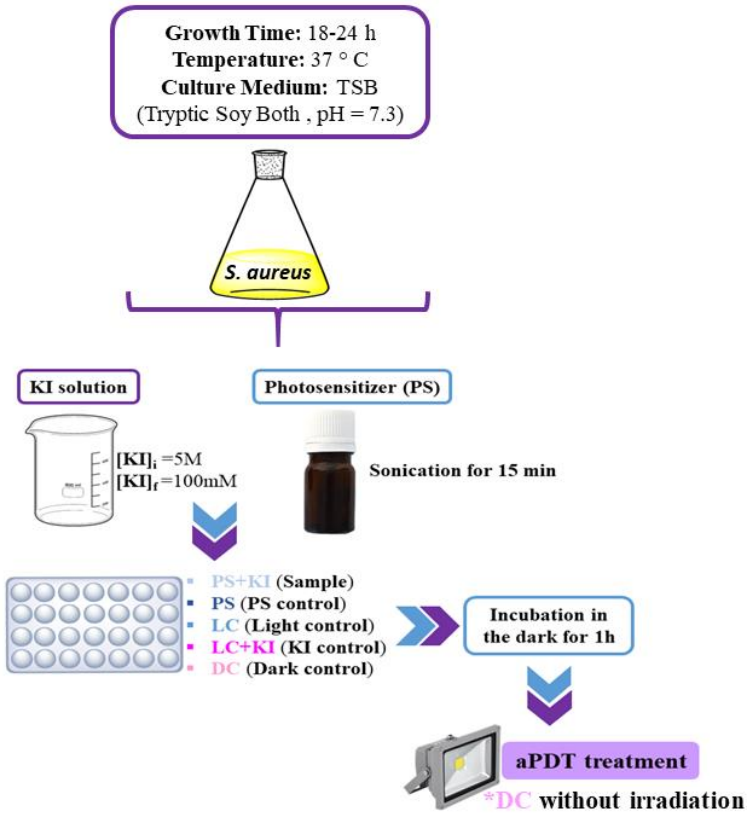



***Figure 1:*** *Schematic representation of the photodynamic assays.*

### 3.10. Statistical

At least three independent experiments with two replicates per assay for each condition were done. The statistical analysis was performed with GraphPad Prism (GraphPad Software, San Diego, USA). Normal distributions were checked by the Kolmogorov–Smirnov test and the homogeneity of variance was verified with the Brown Forsythe test. ANOVA and Dunnet's multiple comparison tests were applied to assess the significance of the differences between the tested conditions. A value of $p < 0.05$ was considered significant.

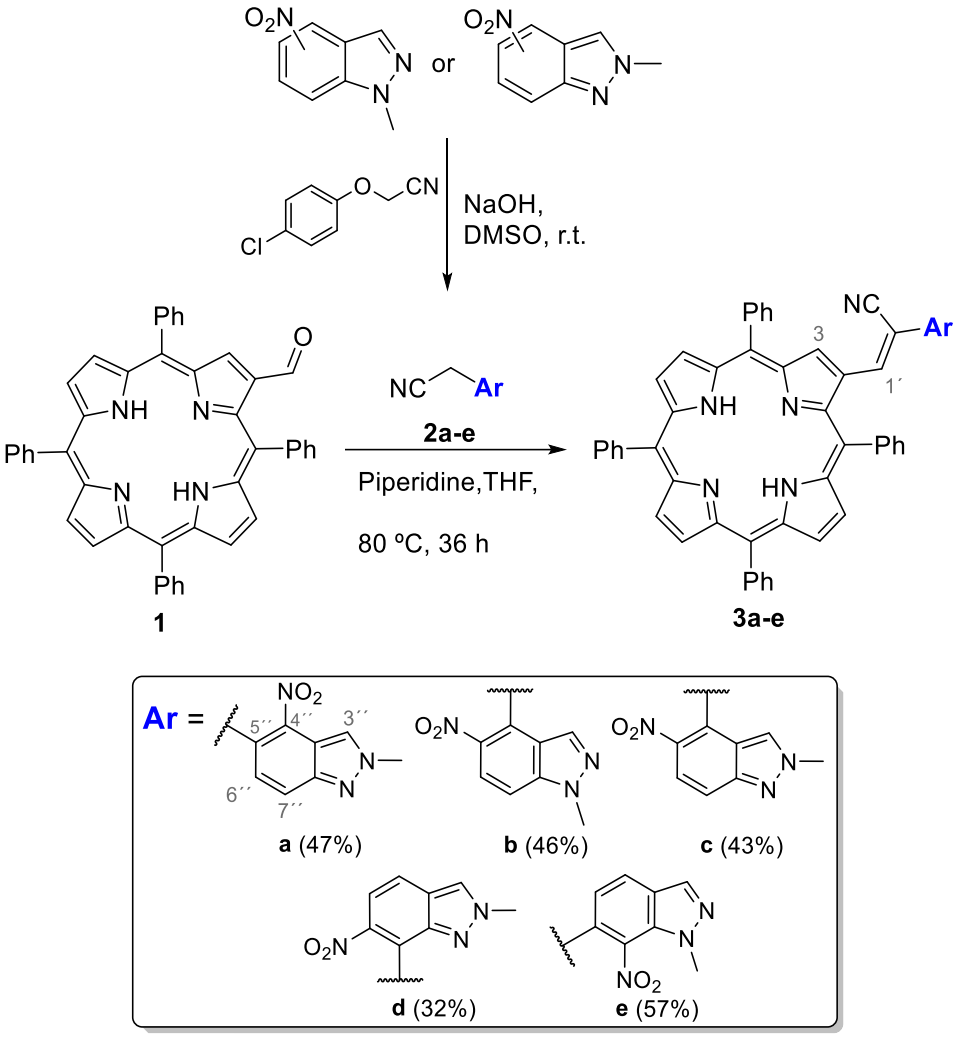
## 4. Results and Discussion

### 4.1. Synthesis and photochemical and photophysical properties

The preparation of the porphyrin-nitroindazole hybrids **3a-e** is outlined in scheme 1 and as it was mentioned involved the Knoevenagel condensation between the 2-formyl-5,10,15,20-tetraphenylporphyrin **1** and the appropriate *N*-methyl-nitroindazolylacetonitriles

**2a-e.** These derivatives were obtained *via* vicarious nucleophilic substitution of the adequate *N*-methyl-nitroindazole with 4-chlorophenoxyacetonitrile accordingly with a synthetic approach recently described by our group [34]. The formyl component was obtained from 5,10,15,20-tetraphenylporphyrin through a well-established sequence involving copper complexation, Vilsmeier formylation, demetallation of the iminium salt, followed by basic hydrolysis [48, 52].

The reactions were performed in the presence of piperidine at refluxing THF for 36 h, when the TLC control showed the presence of the starting porphyrin accompanied by the formation of a main fraction. After the work-up and purification by silica gel column chromatography, the new derivatives were identified as the expected porphyrin-nitroindazole hybrids **3a-e.** The new conjugates were isolated in yields ranging from 32 to 57% and it was possible to recover 19% of the starting porphyrin **1**; attempts to favour the conversion by increasing the reaction time led to the decomposition of the desired products. Also, other attempts to improve the efficacy of these reactions by changing the base (*e.g.* potassium carbonate, potassium hydroxide, ammonium acetate or DBU) or alternatively by using other solvents (*e.g.* a mixture of THF/MeOH (2:1) or toluene) gave rise to a much worse performance like just the recovery of the starting porphyrin or the isolation of the desired products in much lower yields (*e.g.* 20% with DBU).




***Scheme 1:*** *β-functionalization of 2-formyl-tetraphenylporphyrin with N-methyl-nitroindazoles via Knoevenagel condensation.*

The structures of the newly porphyrin-nitroindazole hybrids **3a-e** were established based on its mass spectrum, which shows the $[M+H]^+$ ion peak at *m/z* = 841.3, and NMR studies (see ESI, Figures S1-S26). In the $^1$H NMR spectrum of derivatives **3a-e**, the expected singlet due to the resonance of the β-pyrrolic proton at H-3 appears at ca. $\delta$ 9.7 ppm. The remaining six β-pyrrolic protons generates signals centred at *ca.* $\delta$ 9.0 and $\delta$ 8.7 ppm. A remarkable signal that proves the success of the Knoevenagel condensation is a singlet with a chemical shift ranging from $\delta$ 6.80 and $\delta$ 7.02 ppm due to the resonance of the vinylic proton.

The resonances of the protons from the nitroindazole moiety generate three signals in the aromatic region, two doublets ranging from $\delta$ 8.30 to 7.01 ppm due to the resonance of the two protons from the six-membered ring and a singlet between $\delta$ 8.04 and $\delta$ 8.57 ppm

due to the resonance of the proton from the pyrazolic ring. In the $^{1}$H NMR spectrum of compounds **3d** and **3e** this signal appears embedded in a multiplet with the resonances of the *orto* protons from the *meso* phenyl rings. The resonance of the *N*-methyl protons appears in the aliphatic region as a singlet centred between $\delta$ 4.17 and $\delta$ 4.37 ppm. The singlet at $\delta$ -2.6 ppm is in accordance with the free-base form of the porphyrin-nitroindazole derivatives and it is due to the resonances of the inner core *N*-H protons.

The photophysical characterization of compounds **3a-e** was performed in DMF solution at 298 K. **Figure 2** shows the absorption, emission and excitation spectra of compound **3c** as a representative example of the porphyrin-imidazole derivatives series.

The absorption spectrum shows the typical features of free base porphyrins due to π–π* transitions with the highly intense Soret band due to the $S_0 \rightarrow S_2$ allowed transition at *ca.* 430 nm and four weak Q bands due to the transition from $S_0 \rightarrow S_1$ between 523 and 658 nm [53]. The introduction of the indazole moiety at the β-pyrrolic position of the porphyrinic macrocycle induces a significant red-shift in the Soret band (*ca.* 15 nm) and in the Q bands (*ca.* 10 nm) in all the porphyrin-indazole derivatives UV-Vis spectra relatively to the reference TPP. The perfect resemble between the absorption and the excitation spectra rules out the presence of any emissive impurity.

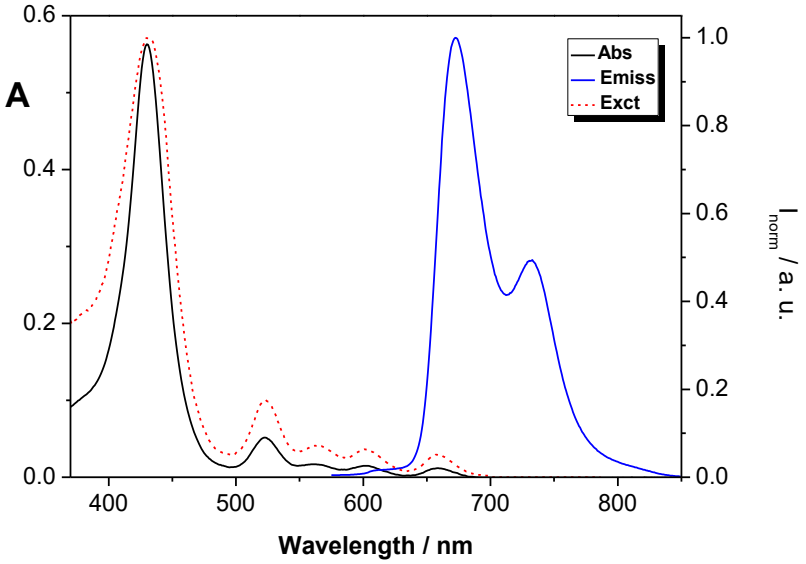



***Figure 2:*** *Absorption (Abs) and normalized emission (Emiss) and excitation (Exct) spectra of compound 3c in DMF at 298 K ([3c] = 5.00 x $10^{-6}$ M, $\lambda_{exc3c}$ = 523 nm and $\lambda_{emiss3c}$ = 731 nm).*

The steady-state fluorescence emission spectra of the porphyrin-indazole derivatives **3a-e** were performed in DMF after their excitation at *ca.* 520 nm (see **Figure 2**). The emission spectra of all the new derivatives present the typical features of *meso*-tetraarylporphyrins, two bands centred in the range between 671-676 nm and at *ca.* 730 nm, where the first vibrational mode of the fluorescence is much more pronounced than the second one. The emission bands can be assigned to $Q_x(0\text{-}0)$ and $Q_x(0\text{-}1)$ transitions, typical of free base porphyrins with a $D_2h$ symmetry due to a nearly unchanged vibronic state upon excitation [54, 55]. The porphyrin-indazole derivatives show low Stokes shifts (12-18 nm), indicating that the spectroscopic energies are similar to the relaxed energies of the lowest singlet excited state $S_1$, which suggest the occurrence of a minor geometric relaxation in the first excited state.

The fluorescence quantum yields ($\Phi_{Flu}$) of the studied compounds **3a-e** were determined by the internal reference method with respect to a solution of 5,10,15,20-tetraphenylporphyrin (**TPP**) used as standard ($\Phi_{Flu}$ = 0.11)[49, 56, 57] and show values slightly lower than the reference, ranging from 0.07 to 0.09, indicating the quenching of the porphyrin excited singlet state by the unit linked into the *beta* position, which probably can be attributed to an alteration of the planarity of the porphyrinic core due to the presence of the indazole moiety that can be responsible for a more reduced π-electron mobility.

In order to evaluate the potential of the new derivatives to be used as PS in antimicrobial photodynamic therapy (aPDT), it was determined their ability in generating $^1O_2$. This efficacy was estimated using 9,10-dimethylanthracene (DMA) as scavenger of the $^1O_2$ produced by the combined action of light, dissolved dioxygen and porphyrin derivative. DMA reacts selectively with the $^1O_2$ generated through a [4+2] cycloaddition reaction affording a non-fluorescent 9,10-endoperoxide species [58]. As reference, it was used **TPP** that was reported as a good oxygen generator ($\Phi_\Delta$ = 0.65) [59].

The results obtained for the ability of porphyrin-indazole derivatives **3a-e** to generate $^1O_2$ are presented in Table S1. In general, all the studied compounds are able to produce singlet oxygen, however, the efficiency of compound **3a** to produce this cytotoxic species is very low when compared with **TPP** ($\Phi_\Delta$ = 0.10) and with the others porphyrin-indazole derivatives **3b-e**. Compounds **3b**, **3d** and **3e** showed to be the best $^1O_2$ generators with a $^1O_2$ quantum yield ranging from $\Phi_\Delta$ =0.45 to $\Phi_\Delta$ = 0.53, these derivatives reach an efficiency

around ≈ 70 to ≈ 80 % of the reference **TPP** in the same light conditions. It was observed that compound **3c** ($\Phi_\Delta$ = 0.32) presents a moderate ability to generate the cytotoxicity species, $^1O_2$, induced a ≈ 50 % lower DMA photooxidation than that caused by **TPP**. No photooxidation of DMA was observed in the absence of a photosensitizer molecule.

The ability demonstrate by the porphyrin-indazole derivatives to generate $^1O_2$ after being exposed to light and in the presence of dioxygen make them good candidates to be used as PS in the photodynamic inactivation of microorganisms.

### 4.2. Detection of Iodine Formation

The detection of iodine formation allows us to elucidate if the photodynamic efficiency of each PS can be enhancement by the presence of KI and if it is related with the antimicrobial iodine formation. In this sense, each compounds at a concentration of 5.0 µM was irradiated with white light in the absence and in the presence of KI at a concentration of 100 mM during pre-defined irradiation times and the absorbance at 340 nm was monitored. The results are represented in **Figure 3**.

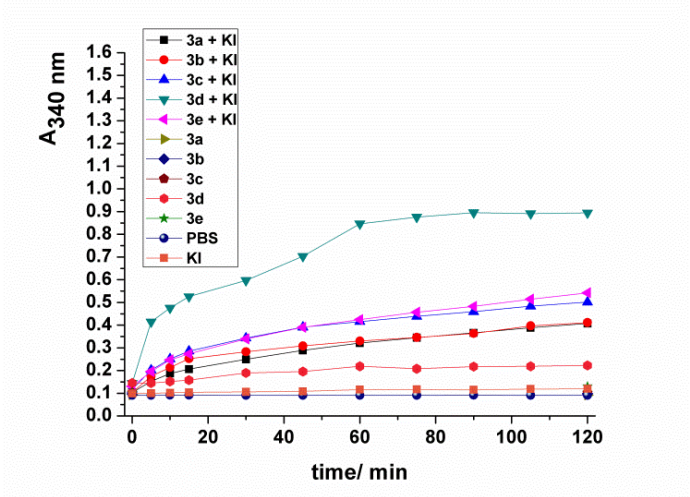



***Figure 3:*** *Monitoring of the generation of iodine, at 340 nm, after different irradiation times in the presence of each compound at 5.0 µM and combinations of each compound at 5.0 µM and KI at 100 mM.*

In the absence of KI, the absorbance at 340 nm remained stable during the 120 min of irradiation, and the same was found for controls. When KI was added to the PS solution, it was observed that compound **3d** showed the highest iodine production during the irradiation procedure, once the absorbance at 340 nm due to the presence of iodine, increase approximately twice comparing with the absorbance of the other compounds, during the first

5 min of irradiation. Compounds **3a**, **3b**, **3c** and **3e** also showed a gradual increase of the absorbance at 340 nm being compounds **3c** and **3e** slightly more effective at producing iodine than the compounds **3a** and **3b**.

### 4.3. Photodynamic inactivation of bacteria

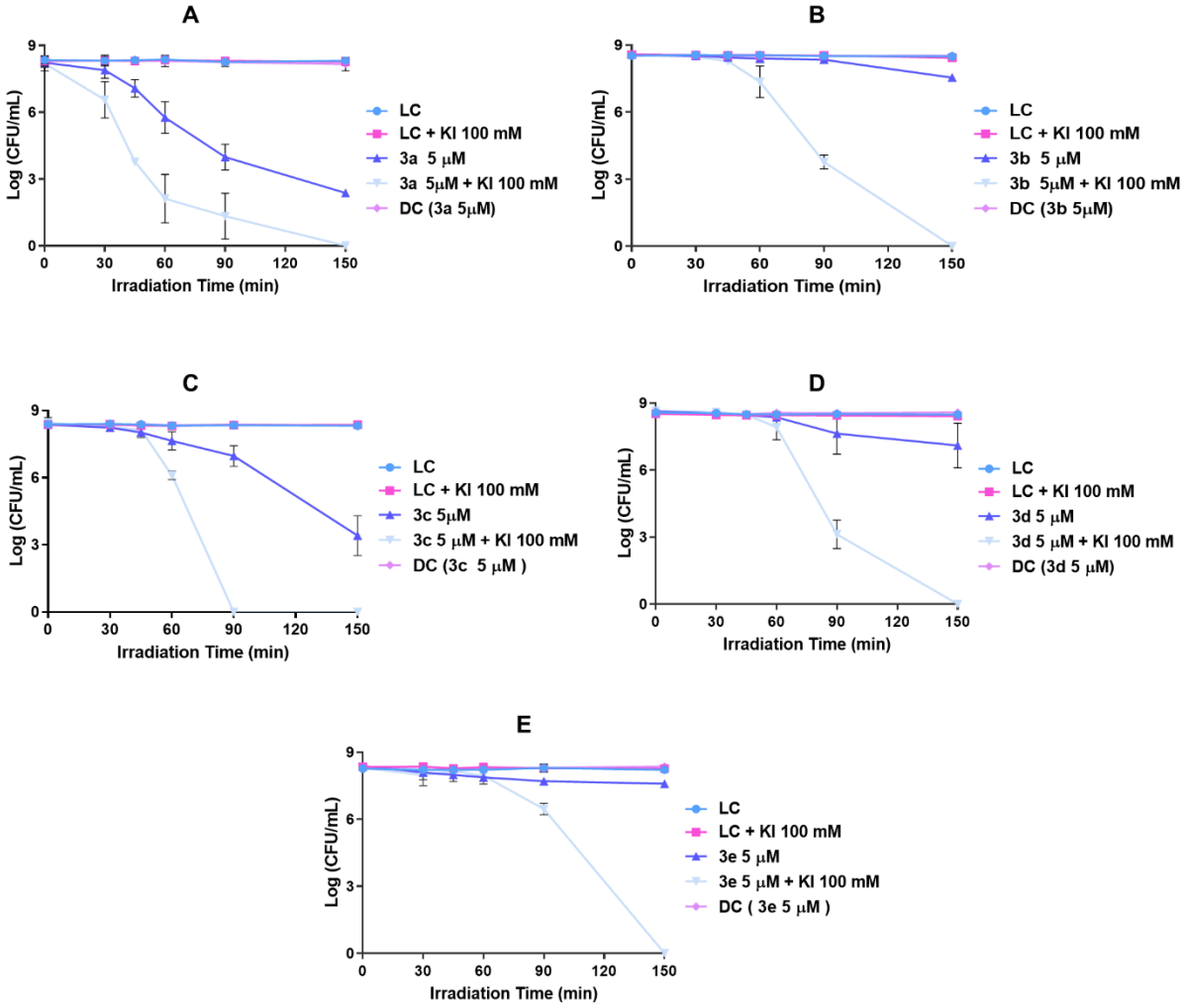



***Figure 4:*** *Photodynamic inactivation of S. aureus MRSA in the presence of **3a** (A), **3b** (B), **3c** (C), **3d** (D) and **3e** (E) at 5.0 μM and 100 mM of KI in PBS and irradiated with white light (380-700 nm) at 50 mW/cm$^2$. Values represent the average of three independent experiments with two replicates each; error bars indicate the standard deviation. Lines just combine the experimental points.*

**Figure 4** represents the photodynamic inactivation profile of *S. aureus* MRSA in PBS using the neutral porphyrins, **3a**, **3b**, **3c**, **3d** and **3e**, at 5.0 μM with and without KI at 100 mM when irradiated with white light (380-700 nm) at 50 mW.cm$^{-2}$. The results showed that the neutral porphyrin **3a** (**Figure 4A**) is effective in the photoinactivation of MRSA, promoting a significant decrease of 5.9 $\log_{10}$ (ANOVA, $p < 0.05$) in the bacterium survival after 150 min of aPDT treatment. One the other hand, the addition of the KI salt improved

the photoinactivation rates promoting a decrease in the survival of the MRSA till the detection limit after 150 min of irradiation (8.3 $\log_{10}$ [ANOVA, $p < 0.05$]). In the cases of the LC (bacteria + light irradiation), LC+KI (bacteria + KI+ light irradiation) and DC (bacteria + PS in the dark) no decrease in MRSA abundance was detected. These results indicate that the viability of this bacterium was not affected by irradiation, the PS in the dark or by the presence of the KI salt in the absence of PS. So, the synergistic effect between the light, PS and KI is the key to the successful aPDT treatment of MRSA.

In **Figure 4B** are summarized the effect of the **3b** in the photoinactivation of MRSA. The results show that PS by itself does not appear to be efficient in the inactivation of *S. aureus*, promoting a small decrease of ~1.0 $\log_{10}$ (ANOVA, $p < 0.05$) after 150 min of treatment. Although this PS did not show satisfactory and promising inactivation rates with the addition of KI it was possible to promote a significant decrease in the survival of the MRSA till the detection limit of the method after 150 min of irradiation (8.5 $\log_{10}$ [ANOVA, $p < 0.05$]). These outstanding results show that the presence of KI during the aPDT procedure appears to greatly improve the photoinactivation rate, allowing that a PS with low photodynamic efficiency is be able to photoinactivate a resistant gram-positive bacteria (MRSA).

The photodynamic effect of porphyrin **3c** was then tested under the same conditions as above and the results are shown in **Figure 4C**. Once more, it seems that the KI salt is a strong aPDT coadjuvant and significantly improve the photodynamic effect of this PS. Moreover, the addition of KI allows increasing MRSA photoinactivation rates and achieving the detection limit of the method after 90 min of treatment (8.3 $\log_{10}$ [ANOVA, $p < 0.05$]). Between the 5 nitroindazole porphyrins analyzed, **3c** proved to be the PS that had a better potentiation by KI, promoting a decrease in the survival of the MRSA till the detection limit of the method 1 hour earlier. Regarding the efficiency of PS alone, it is visualized in **Figure 4C** that **3c** appears to be efficient in photoinactivation of *S aureus* MRSA, promoting a significant decrease of 4.9 $\log_{10}$ (ANOVA, $p < 0.05$) after 150 min of irradiation

Now, focus our attention on the photodynamic efficiency of porphyrin-nitroindazole **3d** (**Figure 4D**) it can be concluded that despite promoting decreases in the order of 0.9 $\log_{10}$ and 1.4 $\log_{10}$ after 90 and 150 min of aPDT treatment, respectively, this was not significant when compared to light controls (ANOVA, $p > 0.05$). It is important to note that the controls

(LC, LC + KI and DC) remained constant throughout treatment confirming the viability of the bacterium. When the KI salt was added to PS there appears to be an ideal synergistic effect increasing the ability of this porphyrin to inactivate the MRSA, allowing a considerable and significant viability decrease of 5.4 $\log_{10}$ (ANOVA, $p < 0.05$) after 90 min of treatment and 8.48 $\log_{10}$ (ANOVA, $p < 0.05$) after 150 min of irradiation, achieving the reduction to the detection limit of the method. In conclusion the salt addition promoted an extraordinary improvement in the inactivation profile, being this improvement gradual throughout the treatment.

The fifth photosensitizer to be analyzed was **3e** at 5.0 μM and 100 mM of KI under the same conditions as for the previous ones. The results are presented in **Figure 4E**. The efficiency of the treatment without the effect of the salt, shows that this PS appears to be inefficient in the photoinactivation of the bacterium, causing a small decrease of 0.6 $\log_{10}$ (ANOVA, $p < 0.05$), similar to that of porphyrin **3b**. Once again, the addition of KI significantly improves the photodynamic effect of this PS, promoting a decrease in the survival of the MRSA till the detection limit of the method after 150 min of irradiation (8.21 $\log_{10}$ [ANOVA, $p < 0.05$]). Although this porphyrin without addition of the KI has an inactivation profile identical to that of porphyrin **3b**, the addition of KI appears to show a more gradual effect in the porphyrin **3b** than in **3e**, since after 90 min of treatment **3b + KI** promote a decrease in the survival of the MRSA of 4.74 $\log_{10}$ (ANOVA, $p < 0.05$) whereas **3e + KI** at the same time did not cause a significant bacterial reduction, by the way, after 90 min of treatment the decrease was 1.83 $\log_{10}$, significantly lower than the decrease obtained with **3b + KI**. However, an abrupt decrease in the survival of the MRSA in the last hour of treatment was observed.

The addition of non-toxic salts such as KI potentiate the action of neutral, anionic and dye photosensitizers. Recent studies have shown that the addition of non-toxic salts to neutral and anionic PSs has been shown to be more relevant against Gram-positive bacteria [44]. By the way, the literature contains reports that the permeability barrier of Gram-negative bacteria is sufficient to protect this bacterial group from anionic and neutral PSs, hindering the interaction of PS with the bacterial membrane that can protect itself from photodynamic action of non-cationic PSs as well as singlet oxygen generated extracellularly [60,61]. Additionally, reports showed that KI was also able to potentiate the aPDT process mediated by some cationic PSs, it is undeniable that the ability of KI to potentiate the aPDT

process, mediated by a broad spectrum of PSs, allows a major decrease of the aPDT treatment time and the reduction of the PS concentration [40]. Having this in mind, we analyzed the antimicrobial properties of new nitroindazole porphyrin conjugates and evaluated the KI ability to potentiate the aPDT process mediated by these neutral porphyrins.

The results showed that porphyrins **3a** and **3c** were the most effective in bacterial photoinactivation promoting decreases in the survival of the MRSA of 5.93 $\log_{10}$ and 4.9 $\log_{10}$, respectively. These photoinactivation rates indicate that these compounds seem to be very promising in the photoinactivation of this resistant Gram-positive bacterium. Regarding to singlet oxygen production, the results show that compound **3a** is the one with the lowest ability to producing singlet oxygen, the compounds **3b**, **3d** and **3e** showed to be the best singlet oxygen generators and finally, the compound **3c** presents a moderate ability to produce singlet oxygen. Contrary to expectations, the porphyrin **3a** despite its low singlet oxygen production, was shown to be the more effective in the photoinactivation of MRSA followed by compound **3c**, which produces more singlet oxygen than **3a** but its ability to generating singlet oxygen is moderate. In other words, the compounds with the highest singlet oxygen production capacity (**3b**, **3d** and **3e**), were surprisingly the least effective in the bacterial photoinactivation. It can be argued that although these compounds produce more singlet oxygen they may have less ability to interact with the bacterial membrane. Knowing that proteins and lipids from the cytoplasmic membrane and bacterial cell wall are the main targets of aPDT, if PS interaction and, consequently its binding to these extracellular structures is lower, the inactivation profile may be negatively affected [62]. In addition, the compounds **3b**, **3d** and **3e** may undergo photodegradation throughout treatment as with methylene blue (**MB**) [63] or even aggregation.

As expected, the addition of KI potentiate the aPDT process mediated by all the analyzed compounds, successfully inactivating MRSA till the detection limit of the method. The results revealed the combined effect between salt and porphyrins increasing the inactivation rates and significantly improving the antimicrobial activity of the less efficient compounds, such as **3b**, **3d** and **3e**. Some reports reveal that peroxyiodide decomposes by two distinct pathways leading to formation of free iodine and hydrogen peroxide (first pathway) and iodine reactive radicals (second pathway). It is known that the reactive iodine radicals promotes a gradual increase in killing rate, one the other hand, the free iodine is the most prevalent specie promoting an abrupt increase in killing rate [40, 43]. **Figure 4** shows

that the addition of KI to compound **3a** caused a gradual decrease in survival of the MRSA, in the case of compounds **3b-3e** this decrease appears to be abrupt. These results seem to be in good agreement with those presented in **Figure 3**, which indicates that compound **3a + KI** has the lowest iodine production capacity, however, the addition of KI still has a beneficial and promising effect on the aPDT process mediated by this porphyrin indicating that despite the lower prevalence of iodine, this compound appears to produce reactive iodine radicals what justifies the gradual inactivation profile shown in **Figure 4A**. Also in **Figure 3** it was possible to conclude that the compound that produces more free iodine was **3d + KI** which justify the abrupt decrease in survival of the MRSA between 60 and 90 min of treatment shown in **Figure 4D** [40].

Vieira et al (2018) showed that in cationic photosensitizers (with 3 or more positive charges) the addition of 100 mM of KI appears to improve the inactivation profile of *E. coli*, a Gram-negative bacteria, allowing to reduce the treatment time and the PS concentration required for the photoinactivation of this bacterium. Additionally, KI potentiation in aPDT process mediated by cationic photosensitizers (**Tri-Py(+)-Me** , **Tetra-Py(+)-Me** and **FORM**) seems to be more effective than that mediated by neutral photosensitizers (**Tetra-Py**). These results indicate that neutral photosensitizers even with the addition of salt does not seem to be the most appropriate for the photoinactivation of Gram-negative bacteria [40]. One the other hand, some reports has shown that neutral photosensitizers and dyes improved by KI action, seems to be efficient in *S. aureus* MRSA photoinactivation, which is supported by our results. Santos et al (2019) showed that the combined use of dyes, **Rose Bengal** and **Eosin**, and KI against MRSA allows a reduction in the PS concentration up to 1000 times [64].

## 5. Conclusions

The Knoevenagel condensation of 2-formyl-tetraphenylporphyrin with a series of *N*-methyl-nitroindazole derivatives showed to be an efficient synthetic access to new porphyrin-indazole hybrid. The ability of these derivatives to generate oxygen singlet and iodine when irradiated in the presence of KI are in accordance with the aPDT studies performed towards *S. aureus MRSA.* According to the American Society of Microbiology, compounds **3a** and **3c** in the absence of KI, are considered good antimicrobials causing decreases in the bacterial survival of 3 or more $\log_{10}$. However, the addition of KI improved significantly the photodynamic efficiency of all compounds allowing a decrease in the

survival of the *S. aureus* MRSA till the detection limit after 150 min (**3a**, **3b**, **3d** and **3e**) and 90 min (**3c**) of aPDT treatment.

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