Paziani, MH, Tonani, L, de Menezes, HD, Bachmann, L, Wainwright, M, Leite Braga, GÚ and von Zeska Kress, MR

Antimicrobial Photodynamic Therapy with Phenothiazinium Photosensitizers in non-vertebrate model Galleria mellonella infected with Fusarium keratoplasticum and Fusarium moniliforme.

Title: Antimicrobial Photodynamic Therapy with Phenothiazinium Photosensitizers in non-vertebrate model *Galleria mellonella* infected with *Fusarium keratoplasticum* and *Fusarium moniliforme*.

Authors: Mario Henrique Paziani[1], Ludmilla Tonani[1], Henrique Dantas de Menezes[1], Luciano Bachmann[2], Mark Wainwright[3], Gilberto Úbida Leite Braga[1], Marcia Regina von Zeska Kress[1]

Corresponding author: Marcia Regina von Zeska Kress, Av. do Café s/n, CEP 14040-903, Ribeirao Preto, São Paulo, Brazil. e-mail kress@fcfrp.usp.br

1 Departamento de Analises Clinicas, Toxicologicas e Bromatologicas, Faculdade de Ciencias Farmaceuticas de Ribeirao Preto, Universidade de Sao Paulo, Ribeirao Preto, SP 14040-903, Brazil

2 Departamento de Fisica, Faculdade de Filosofia, Ciencias e Letras de Ribeirao Preto, Universidade de Sao Paulo, Ribeirão Preto, SP 14040-901, Brazil.

3 School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Liverpool L3 3AF, United Kingdom.

ABSTRACT

Fusarium keratoplasticum and *Fusarium moniliforme* are filamentous fungi common in the environment and cause mycosis in both animals and plants. Human infections include micetomas, keratitis and onychomycosis, while deeper mycosis occurs in immunocompromised patients. Most of the *Fusarium* spp. are frequently resistant to treatment with currently used antifungals. The frequent occurrence of antifungal resistance has motivated the study of antimicrobial photodynamic therapy as an alternative treatment for fungal infections. Many studies have investigated the *in vitro* use of antimicrobial photodynamic therapy to kill fungi, but rarely in animal models of infection. Thus, here we employed the invertebrate wax moth *Galleria mellonella* to study the *in vivo* effects of antimicrobial photodynamic therapy with three different phenothiazinium photosensitizers, methylene blue, new methylene blue N and the pentacyclic S137 against infection with microconidia of *Fusarium keratoplasticum* and *Fusarium moniliforme*. The effect of antimicrobial photodynamic therapy using these photosensitizers and light-emitting diodes with an emission peak at 635 nm and an integrated irradiance from 570 to 670 nm of 9.8 mW cm^{-2} was investigated regarding the toxicity, fungal burden, larval survival and cellular immune response. The results from this model indicate that antimicrobial photodynamic therapy with methylene blue,

new methylene blue N and S137 is efficient for the treatment of infection with *F. keratoplasticum* and *F. moniliforme*. The efficiency can be attributed to the fungal cell damage caused by antimicrobial photodynamic therapy which facilitates the action of the host immune response.

Keywords: antimicrobial photodynamic therapy; *Fusarium keratoplasticum; Fusarium moniliforme; Galleria mellonella;* mycosis.

1. Introduction

Photodynamic inactivation is a promising strategy to use in local infection control. It combines the interaction of a photosensitizer (PS), light and molecular oxygen. Photosensitizers are intentionally non-toxic molecules that are able to absorb and transfer energy or electrons after light absorption to molecular oxygen for the generation of reactive oxygen species (ROS), mainly singlet oxygen, which oxidize nonspecific microbial targets (Wainwright et al., 2017; Zhou et al., 2018). The advantages of antimicrobial photodynamic inactivation are double selectivity (light and PS localization), the very unlikely development of microbial resistance and efficient activity in multi-resistant strains (Hamblin and Hasan, 2004; Wainwright et al., 2017).

The PSs initially proposed for antimicrobial use were phenothiazinium dyes (Bhatti et al., 1998; Wainwright et al., 1998; Gad et al., 2004), perylenequinones (Kubin et al., 1999) and rose bengal (Schafer et al., 2000), , and. The first phenothiazinium dyes, thionin and methylene blue (MB), were described for the staining of specimens in biomedical microscopy. The versatile MB is a well-known cationic phenothiazinium dye and has been used and approved for clinical treatment (e.g. dementia, cancer and antimicrobial chemotherapy) (Dai et al., 2012; Wainwright et al., 2017). Despite the enormous existing confidence in commercial PS, the study of new molecules, including new derivatives of MB, offers great improvements in antimicrobial use (Wainwright, 2009; Wainwright 2018; Wainwright et al., 2016; Wainwright and Crossley, 2002). New methylene blue (NMBN) is a close derivative of MB and was initially used for the routine staining of reticulocytes (Bain et al., 2017). NMBN is an active photoantimicrobial *in vitro* against *Trichophyton* sp. (Rodrigues et al., 2012), and *Candida* species (Rodrigues et al., 2013), and is a viable approach for prophylaxis and treatment of cutaneous infections caused by *C. albicans* (Dai et al., 2011). S137, also named DO15 (1,11-diethyl-2,2,4,8,10,10-hexamethyl-1,2,3,4,8,9,10,11-octahydrodipyrido[3,2-*b*:2',3'-*i*]phenothiazinium hydrogensulphate), is a pentacyclic phenothiazinium derivative of MB based on suitable tetrahydroquinolines (Wainwright et al., 2005; Wainwright et al., 2011). This MB derivative was studied *in vitro* for photoinactivation of bacteria and fungi. S137 is effective against the Gram-positive bacterium *Propionibacterium acnes* (Wainwright et al., 2012), and fungal species of the genera *Trichophyton* (Rodrigues et al., 2012), *Candida* (Rodrigues et al., 2013), *Colletotrichum* (de Menezes et al., 2014), *Fusarium* (de Menezes et al., 2016), and *Neoscytalidium* (Tonani et al., 2018).

Fusarium spp. are filamentous fungi easily dispersed in the environment via direct contact, water, wind and vectors. The fungal genus comprises several species that are well known plant and opportunistic human pathogens. Reports of human infections are growing due to both increasing numbers of immunocompromised patients and improved identification in the clinical laboratory (Al Hatmi et al., 2016). The most common clinically-important species is *Fusarium solani* species complex (e.g., *F. falciforme*, *F. keratoplasticum* and *F. petroliphilum*), followed by *F. oxysporum, F. moniliforme* (synonym *F. verticillioides* (Sacc.) Nirenberg), *F. dimerum*, and *F. proliferatum* (Al-Hatmi et al., 2016). Mycosis in humans, such as mycetoma (Katkar et al., 201, Bonifaz et al., 2018), keratitis (Homa et al., 2013) and onychomycosis (Guilhermetti et al., 2007) occurs in immunocompetent patients after traumatic introduction of *Fusarium* spp. Additionally, these species are associated with opportunistic infections in humans (e.g. neutropenic patients), causing localized infections in the skin and other body parts as well as systemic infections which are often difficult to treat and consequently associated with high mortality rates (Nucci and Anaissie, 2007). Difficulties in treatment are attributed to primary (intrinsic) and secondary resistance profiles of *Fusarium* spp. to currently used antifungals (Al Hatmi et al., 2016 and 2017).

The antimicrobial properties of drugs have been studied by employing insect models of microbial infections such as the greater wax moth *Galleria mellonella* (Lionakis and Kontoyiannis, 2012). *G. mellonella* reproduces most of the aspects of mammalian infection and their larvae are increasingly used as an alternative model to study pathogenesis and virulence factors of several bacterial and fungal human pathogens. Among the advantages of employing *in vivo* testing with *G. mellonella* are the adaptation to mammalian temperature (37°C); a well-characterized phagocytic system and immune repertoire; large numbers of larvae can be easily and inexpensively obtained in the lab, and no ethical considerations are required (Kelly and Kavanagh, 2011; Giannouli et al., 2014). The immune system of *G. mellonella* has structural and functional similarities to the innate immune response of mammals. The insect hemolymph contains circulating hemocytes (immune cells) with the capacity to immobilize and/or kill microorganisms (Tsai et al., 2016).

Here we investigate the *in vivo* effects of APDT with three different phenothiazinium PSs, MB, NMBN and S137 in the non-vertebrate model host *Galleria mellonella* larvae infected with microconidia of *Fusarium keratoplasticum* (*F. solani* species complex) and *Fusarium moniliforme*.

2. Methods

2.1. Fungal strain, culture conditions and fungal inocula preparation

The strains used in this study were *Fusarium keratoplasticum* Geiser et al. (*Fusarium solani* species complex) (ATCC®36031™) and *Fusarium moniliforme* Sheldon, anamorph (ATCC®38159™) both from the American Type Culture Collection (ATCC) (Manassas, VA, USA). *Fusarium* spp. were inoculated on Potato Dextrose Agar (PDA) media culture (Becton, Dickinson Company, USA) and incubated in the dark at 28ºC for 5 days. Microconidia were collected with sterile phosphate buffered saline (PBS), pH 7,4 and filtered with sterile Miracloth (Merck Millipore). Microconidia were counted using a hemocytometer and the suspension was diluted in sterile PBS.

2.2. *In vitro* antifungal susceptibility test

The tests were conducted according to the Clinical Laboratory Standards Institute (CLSI) reference method for broth dilution antifungal susceptibility testing of filamentous fungi (M38-A2) (Clinical and Laboratory Standards Institute, 2008). The antifungal drugs amphotericin B (AMB - 0.06 to 32 mg mL^{-1}), fluconazole (FLU - 0.12 to 64 mg mL^{-1}), voriconazole (VOR - 0.03 to 16 mg mL^{-1}), and posaconazole (POS - 0.12 to 64 mg mL^{-1}) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). The experiments were performed in 96-well microtitre plates (TPP, Switzerland) with RPMI 1614 culture medium (Gibco, Invitrogen Corporation, NY, USA) buffered with 0.165 M 3-(N-morpholino) propanesulfonic acid (MOPS), pH 7.0, addition of antifungal drugs and 1×10^4 microconidia mL^{-1}. After 48 h of incubation at 28ºC, the minimal inhibitory concentration (MIC) was visually determined. The MIC endpoint was defined as the minimal concentration of the drug capable of inhibiting 100% of fungal growth for AMB and inhibit 90% of fungal growth for azoles. The standard quality control strain of the experiment was *Aspergillus flavus* (ATCC®204304™).

2.3. Photosensitizers and light source

The phenothiazinium photosensitizers (PSs) methylene blue (MB) and new methylene blue N (NMBN) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). The pentacyclic S137 was synthesized as previously described (Wainwright et al., 2011). The solutions and respective dilutions were prepared with sterile PBS. Stock solutions, chemical structures and light source (array of 96 light-emitting diodes with the emission peak at 635 nm and integrated irradiance from 570 to 670 nm of 9.8 mW cm^{-2}) were previously described (de Menezes et al., 2016). The fluence of 15 J cm^{-2} was achieved after 19.5 minutes of light exposure.

2.4. *Galleria mellonella* as an non-vertebrate model for infection, toxicity and antimicrobial therapy tests

Groups of 10 *G. mellonella* larvae were randomly divided to determine the infective inocula of *F. keratoplasticum* and *F. moniliforme*, toxicity of phenothiazinium PSs MB, NMBN, TBO and S137 and APDT efficacy in infections with microconidia of *F. keratoplasticum* and *F. moniliforme*. Additionally, fungal burden and cellular immune response were determined after APDT. *G. mellonella* larvae at the sixth instar of development were selected at 200 ± 50 mg each and an absence of cuticle pigmentation. A Hamilton syringe was used to inject 5 µL inoculum containing PBS (control), phenothiazinium PS and/or 10^4 microconidia of *Fusarium* spp. into the hemocoel of each larvae through the last right proleg. The larvae were exposed to dark (L-) or light (L+) in 24-well culture plate (Costar Corning, New York, NY, USA). After this, the larvae were maintained in glass containers in darkness at 37°C and monitored for survival daily. Survival curves and statistical analysis (Log-rank test, Mantel-Cox) were performed using GraphPad Prism 5 statistical software. *P* value <0.05 was considered statistically significant.

The infective inocula of 10^1, 10^2, 10^3, 10^4 and 10^5 microconidia of *F. keratoplasticum* and *F. moniliforme* were tested in *G. mellonella* larvae with 5 µl injection of 2×10^3, 2×10^4, 2×10^5, 2×10^6 and 2×10^7 microconidia suspension, respectively. The control group consisted of sterile PBS injected larvae. The experiments were repeated twice and representative experiments are presented.

The toxicity of phenothiazinium PSs in *G. mellonella* were tested based on minimum fungicidal concentrations (MFC) determined by De Menezes et al. (2016). Given the PS distribution in the larvae, the concentrations were converted into molality (mol Kg^{-1}) where the total mass consisted of the weight of the larvae (200 mg). The toxicity of MB was tested in *G. mellonella* at a final working concentration of 750, 1500 and 3000 µM. The final working concentration of NMBN and S137 were 100, 200 and 400 µM. Five microliters from the working concentration of each PS were injected in groups of *G. mellonella* larvae and the concentration of MB in each larva was, respectively, 18.75, 37.5 and 75 µmol Kg^{-1}. The concentration of NMBN and S137 were, respectively, 2.5, 5 and 10 µmol Kg^{-1}. The control group consisted of sterile PBS injected larvae. After PS injection, larvae were maintained in darkness or exposed to light (15 J cm^{-2}). The experiments were repeated twice and representative experiments are presented.

APDT with phenothiazinium PSs was evaluated in *G. mellonella* infected with *F. keratoplasticum* and *F. moniliforme*. The final concentration of microconidia injected in

each larva was 10^4 microconidia (5 µl of 2 × 10^6 microconidia mL^{-1}). The final working concentration of phenothiazinium PSs were 750 and 1500 µM for MB, and 100 and 400 µM for S137 and NMBN. The microconidial suspension was prepared in PBS or in the PS solution in which both microconidia and PS were injected together in 5 µl inoculum. The control group consisted of sterile PBS-injected larvae. Immediately after injection, the groups of larvae were maintained in darkness or exposed to light (15 J cm^{-2}). The experiments were repeated twice and representative experiments are presented.

The number of viable microconidia (fungal burden) and total hemocyte count (THC) were analyzed after APDT with MB (1500 µM), NMBN (200 µM) and S137 (200 µM) either maintained in darkness or exposed to light (15 J cm^{-2}). After APDT the larvae were incubated 30 minutes in the dark and five larvae per group were bled using a sterile lancet into the hemocoel and the hemolymph was collected.

The fungal burden was determined by the serial dilution of the hemolymph and inoculation on PDA supplemented with 0.12 mg mL^{-1} deoxycholic acid and 50 µg mL^{-1} chloramphenicol. The numbers of colony-forming units (CFU) were counted after 48 h of incubation at 30ºC. The experiments were repeated twice and representative experiments are presented.

The THC in the hemolymph of *G. mellonella* larvae were assayed using a protocol from Blanco et al. (2017). The experiment was performed with 3 larvae for each test condition. The hemolymph was collected and diluted in cold IPS (150 mM sodium chloride, 5 mM potassium chloride, 100 mM Tris hydrochloride ph 6.9, 10 mM EDTA and 30 mM sodium citrate). The hemocyte density was assessed with hemocytometer and observed under a light microscope (Coleman with 40× objective). The experiments were repeated twice and representative experiments are presented.

3. Results

3.1 Antifungal susceptibility test

The antifungal susceptibility of *F. keratoplasticum* and *F. moniliforme* were tested to the polyene amphotericin B and three azoles, voriconazole, posaconazole and itraconazole. *Aspergillus flavus* was the control strain of the antifungal susceptibility experiment. The MIC values of the quality control strain were reproducible and fell within the established ranges published by CLSI methodologies (Clinical and Laboratory Standards Institute, 2008). The analysis revealed that itraconazole and posaconazole exhibited *in vitro* activity against *F. keratoplasticum* and *F. moniliforme* (MIC value 32 to > 64 µg mL^{-1}). Voriconazole and amphotericin B were active with MIC ranges of 4 to 8 µg mL^{-1} and 1 to 2 µg mL^{-1}, respectively (table 1). Therefore, the high

MIC values of itraconazole and posaconazole required to inactivate *F. keratoplasticum* and *F. moniliforme* indicate that these strains are resistant to these antifungal agents.

3.2 Infective inoculum effect of *F. keratoplasticum* and *F. moniliforme* microconidia in *G. mellonella* larvae survival

Microconidial inocula ranging from 10^1 to 10^5 microconidia/larva were used to investigate the virulence of *F. keratoplasticum* and *F. moniliforme* against *G. mellonella* (Figure 1). Inocula of 10^1, 10^2 and 10^3 microconidia/larva of *F. keratoplasticum* and 10^1 microconidia/larva of *F. moniliforme* did not reduce larval survival until 9 days post-infection compared to the uninfected control (PBS). However, inocula of 10^4 and 10^5 microconidia/larva of *F. keratoplasticum* and 10^2 to 10^5 microconidia/larva of *F. moniliforme* reduced larval survival compared with lower inocula and uninfected controls (PBS). Therefore, larval survival was affected by the microconidial inoculum in a dose-dependent manner during 9 days incubation (Figure 1). The inoculum of 10^4 *F. moniliforme* microconidia/larva was able to kill 100% of the larvae at 4 days post-infection. The same inoculum of *F. keratoplasticum* killed 100% of the larvae at 8 days post-infection ($P \leq 0.05$ - Figure 1). The inoculum of 10^4 microconidia/larva was considered optimum for the production of acute infection with *F. keratoplasticum* and *F. moniliforme* in *G. mellonella* larva.

3.3 Toxicity of APDT with phenothiazinium PSs to *G. mellonella* larvae

The toxicity of phenothiazinium PSs MB, NMBN and S137 in darkness or with exposure to light (15 J cm^{-2}) was tested in *G. mellonella* larvae. The exposure to light alone (without PS) at 15 J cm^{-2} did not affect larval survival. Similarly, treatment with NMBN (100, 200 and 400 µM) both in darkness and with light at 15 J cm^{-2} did not affect this. S137 (100, 200 and 400 µM) produced low mortality of the larvae (10 to 20%) in both darkness and with light. APDT with MB (750 and 1500 µM) killed only 10% of the larvae. However, the working concentration of 3000 µM of MB led to 50% mortality after 8 days (Figure 2). So, the phenothiazinium PSs MB, NMBN and S137 at the working concentration of 1500 µM, 200 µM and 200 µM, respectively, and exposure to 15 J cm^{-2} were not toxic or presented low toxicity to *G. mellonella* and could be used in the treatment of *Fusarium* spp. infections.

3.4 The effect of APDT with phenothiazinium PSs in *G. mellonella* infected with *F. keratoplasticum* and *F. moniliforme*

The effect of APDT with MB, NMBN and S137 was evaluated in larvae of *G. mellonella* infected with 10^4 microconidia of *F. keratoplasticum* and *F. moniliforme*. Both species were able to kill 100% of the larvae, respectively, by the 5th and 7th days, respectively,

after microconidial injection (Figure 1). The injection of MB (1500 μM), NMBN (200 μM) and S137 (200 μM) in darkness did not prevent the death of most larvae infected with *F. keratoplasticum* and *F. moniliforme* (Figure 3 and 4). APDT was performed with two different concentrations of each PS and exposure to 15 J cm^{-2}. The survival of infected larvae with *F. keratoplasticum* were 60, 20 and 40% after APDT with MB (750 μM), NMBN (100 μM) and S137 (100 μM), respectively. *F. moniliforme* infected larvae demonstrated 20, zero and 90% survival after APDT with MB (750 μM), NMBN (100 μM) and S137 (100 μM), respectively. At higher concentrations of PS, *F. keratoplasticum* infected larvae survival levels were 70, 60 and 80% after APDT with MB (1500 μM), NMBN (200 μM) and S137 (200 μM), respectively. The survival levels of larvae infected with *F. moniliforme* were 40, 10 and 100% after APDT with MB (1500 μM), NMBN (200 μM) and S137 (200 μM), respectively. Therefore, the survival of infected larvae using APDT was improved by increasing the PS working concentrations to 1500 μM (MB), 200 μM (NMBN) and 200 μM (S137) (Figure 3 and 4). APDT with S137 was the most effective for both fungal species.

3.5 Fungal burden in *G. mellonella* larvae after APDT with phenothiazinium PSs

F. keratoplasticum and *F. moniliforme* burdens were determined in the hemolymph of infected larvae of *G. mellonella* after APDT with MB, NMBN and S137. Neither exposure only to light (15 J cm^{-2}) nor treatment only with the PSs MB (1500 μM), NMBN (200 μM) and S137 (200 μM) killed the microconidia of *F. keratoplasticum* and *F. moniliforme* in infected larvae. Conversely, APDT with MB, NMBN and S137 in *G. mellonella*-infected larvae reduced the survival of *F. keratoplasticum* microconidia to 38, 57 and 2%, respectively. The survival levels of *F. moniliforme* microconidia in infected-larvae were 69, 37 and 21%, respectively (Figure 5). This indicates that APDT with MB, NMBN and S137 in *G. mellonella*-infected larvae are able to significantly reduce the viability of *F. keratoplasticum* and *F. moniliforme* microconidia *in vivo*.

3.6 Total hemocyte count of *G. mellonella* larvae after infection and APDT

We investigated the total hemocyte count (THC) in the hemolymph of *G. mellonella* larvae in response to the infection with microconidia of *F. keratoplasticum* and *F. moniliforme*, and APDT with MB, NMBN and S137. The THC in naïve larvae (control group) was 137/μL and increased to 253/μL and 273/μL in PBS-injected larvae in darkness and exposed to light (15 J cm^{-2}), respectively. APDT (15 J cm^{-2}) with the PS MB, NMBN and S137 in non-infected larvae has shown THCs greater than in non-infected larvae with the same PS in dark conditions (Figure 6). Larvae infected with *F. keratoplasticum* and *F. moniliforme* had THCs of 159/μL and 48/μL, respectively. Once

the larvae was infected with microconidia of *F. keratoplasticum* and *F. moniliforme*, APDT (15 J cm^{-2}) with MB, NMBN and S137 generally increased the THC, in comparison to the same treatment in dark conditions (Figure 06). The only exception was the decrease of THC in the APDT (15 J cm^{-2}) with NMBN in larvae infected with *F. keratoplasticum* (figure 06). Regardless of the THC decreasing, the larval survival remained high after APDT (Figure 3). Thus, the results indicate that the inoculation of PBS in the larval hemolymph stimulates hemocyte proliferation. It is clearly shown that *F. moniliforme* poorly induces hemocyte proliferation in comparison to *F. keratoplasticum*, demonstrating that the cellullar immune stimulus depends on the species of the microorganism. Finally, the overall activity of APDT with MB, NMBN and S137 increases the THC in the hemolymph of both infected and non-infected larvae.

Discussion

In this study we evaluated APDT with two commercial (MB and NMBN) and one novel (S137) phenothiazinium PS in the *in vivo* model *Galleria mellonella* infected with microconidia of *F. keratoplasticum* and *F. moniliforme*.

First we determined the virulence of *F. keratoplasticum* and *F. moniliforme* in *G. mellonella*. Different inocula of fungal microconidia were injected in the *G. mellonella* larvae. Based on the results, we observed that the virulence level of both species is dependent on the concentration of the microconidial inoculum. Additionally, we concluded that *F. moniliforme* is more virulent than *F. keratoplasticum*, showing that there are differences in virulence regarding strains and/or species. In a pathogenesis study, *Fusarium* species were able to kill *Galleria mellonella* larvae in a factor-dependent manner, such as fungal strain, incubation temperature after larvae inoculation, and conidial morphology (Coleman et al*.,* 2011). Clinical isolates of *F. solani* species complex have shown high virulence in a murine disseminated-infection model, while avirulence was presented by other species including *F. verticillioides* (= *F. moniliforme)* (Coleman et al., 2011). Therefore, the virulence of *Fusarium* species and strains to *G. mellonella* is surely dependent on diverse factors such as species, strains, temperature, host, conidial morphology and microconidial inoculum dose. Notwithstanding, we determined a lethal concentration of *F. moniliforme* and *F. keratoplasticum* microconidia against *G. mellonella* to study the effects of APDT in experimental infection assay.

Fusarium spp. are considered multi-resistant pathogens (Baltazar et al*.*, 2015) and are resistant to most antifungal agents (Nucci and Anaissie, 2007). Our results demonstrate the resistance of *F. keratoplasticum* and *F. moniliforme* to azoles. Indeed,

this resistance underlines the urgency of investigating new antifungal therapies. Antimicrobial photodynamic therapy (APDT) has proved to be an effective alternative approach to control fungal infections (Baltazar et al., 2015). The effect of APDT is the production of reactive oxygen species (ROS) which are highly reactive against fungal biomolecules (Hamblin and Hasan, 2004). Since many different fungal biomolecules are potentially damaged, the selection of resistant strains is greatly reduced, making APDT is promising therapeutic alternative against fungal infections. We tested the *in vivo* capacity of APDT with MB, NMBN and S137 by using larvae of the greater wax moth *G. mellonella* infected with microconidia of *F. keratoplasticum* and *F. moniliforme.* This model of infection has the potential to facilitate the *in vivo* study of pathogen-host cross talk (Chibebe Junior et al., 2013). We previously showed the *in vitro* efficacy of APDT with MB, NMBN and S137 against microconidia of *F. keratoplasticum* (*F. solani* species complex) and *F. moniliforme* (de Menezes et al., 2016). Our data indicated that treatment with MB (1500 μM), NMBN (200 μM) and S137 (200 μM) was not toxic to *G. mellonella* larvae. We showed that APDT with MB (1500 μM), NMBN (200 μM) prolonged and increased the survival of *G. mellonella* larvae infected with *F. keratoplasticum* and *F. moniliforme.* So far, this is the first study of APDT with MB, NMBN and S137 in *G. mellonella* larvae infected with microconidia of the filamentous fungi *Fusarium* spp. APDT with MB was studied in *G. mellonella* infected with the yeast fungus *Candida albicans* (Chibebe Junior et al., 2013) and the Gram-negative bacterium *Porphyromonas gingivalis* (Santos et al., 2017), and were able to prolong the survival of the infected larvae. MB and NMBN were effective in APDT of immunosuppressed mouse model of oral candidiasis infection (Freire et al., 2016). MB is often used since was the first antimicrobial PS clinically approved to human use (Dai et al., 2012). Skin ulcers infected with *Fusarium* spp. were successfully treated with MB and red light (Aspiroz et al., 2017). Therefore, APDT with MB and NMBN is effective in different hosts against different infecting microorganism, including azole-resistant strains of the filamentous fungi *F. keratoplasticum* and *F. moniliforme*.

The production of new, potent and clinically compatible PSs is of increasing interest (Dai et al., 2012; Wainwright et al., 2017). We showed the *in vitro* effectiveness of the novel pentacyclic phenothiazinium PS S137 (de Menezes et al., 2016), and this is extended in the current work to demonstrate the *in vivo* effectiveness of APDT with S137 in *G. mellonella* larvae infected with microconidia of both *F. keratoplasticum* and *F. moniliforme*. S137 alone and APDT with S137 were not toxic to *G. mellonella*. The larvae presented a high survival rate after APDT with S137, indicating S137 as an

excellent PS for photodynamic therapy against filamentous fungi, such as the azole resistant strains of *Fusarium* spp.

Organism survival after antifungal treatment depends on different factors inherent to the microorganism and host. The first includes the fungal cell damage caused by the treatment. The second includes the innate immune response (Bergin et al., 2003). The effect of APDT is the production of reactive oxygen species (ROS) which results in oxidative damage to the fungal biomolecules such as lipids, proteins and DNA. This damage usually reduces the viability of the cell (Hamblin and Hasan, 2004; Rosseti et al., 2014). Our group previously showed the *in vitro* damage caused by APDT with toluidine blue (TB), MB, NMBN and S137 to the cell permeability and lipids of *F. keratoplasticum* and *F. moniliforme* microconidia (de Menezes et al., 2016) and *Neoscytalidium* spp. arthroconidia (Tonani et al., 2018). Additionally, the *in vitro* killing effect was demonstrated (de Menezes et al., 2016), and is emphasized by the reduced microconidial viability reported here *in vivo* in *G. mellonella* larvae infected with *F. keratoplasticum* and *F, moniliforme*. Similar results were observed in *G. mellonella* infected with *C. albicans*, where APDT with MB decreased the yeast cell viability immediately after light exposure (Chibebe Junior et al., 2013). Additionally, the production of ROS and consequent cell permeability is increased *in vitro* in *C. albicans* upon APDT with TB (Rosseti et al., 2014). Thus, our results indicate that APDT with MB, NMBN and S137 reduced the microconidia viability due to fungal damage caused by the photochemical products of the treatment.

An important factor to be considered for the survival of an organism is the immune system response to a microorganism invasion. In addition to monitoring the changes in the fungal load, changes in hemocyte density indicate fungal pathogenicity (Bergin et al., 2003). The cellular immune system response (circulating hemocyte) of *G. mellonella* larvae infected with microconidia of the mildly-virulent strain *F. keratoplasticum* was higher than the response of the larvae infected with the highly-virulent *F. moniliforme*. The strains with high pathogenicity succeeded in dealing with the cellular immune response of the larvae which leads to the decrease in hemocyte count and increase in fungal development in the hemolymph. Thus, reduced hemocyte count is correlated to low larval survival and increase in hemocyte count is correlated to high larvae survival.

We also evaluated the effect of APDT on the total hemocyte count in the hemolymph of non-infected and infected *G. mellonella* larvae. Overall, the total hemocyte count after APDT with MB, NMBN and S137 increased in larvae hemolymph whereas the fungal

burden was decreased. Similar results were found in APDT with MB against *Porphyromonas gingivalis* (Santos et al., 2017) and *C. albicans* (Chibebe Junior et al., 2013). The increase in the cellular immune response here is correlated to the increase of larval survival and decrease of fungal burden. Thus, the infected larva survived because the cellular immune system response of *G. mellonella* acted effectively against fungal cells damaged by APDT with MB, NMBN and S137.

Conclusion

F. keratoplasticum and *F. moniliforme* are resistant to itraconazole (MIC 32 µg mL^{-1}) and posaconazole (MIC > 64 µg mL^{-1}). Antimicrobial photodynamic therapy with methylene blue (1500 µM), new methylene blue (200 µM), and the pentacyclic derivative S137 (200 µM) are not toxic or present low toxicity to *G. mellonella* larvae and increase the survival of larvae infected with microconidia of both *F. keratoplasticum* and *F. moniliforme*. Increased larval survival was due to decreased inherent fungal burden and increased cellular immune response.

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References

Al-Hatmi AMS, Meis JF, de Hoog GS. Fusarium: Molecular Diversity and Intrinsic Drug Resistance. PLoS Pathog. 2016, 12(4): e1005464. doi:10.1371/journal.ppat.1005464.

Al-Hatmi AMS, Curfs-Breuker I, de Hoog GS, Meis JF and Verweij PE. Antifungal Susceptibility Testing of Fusarium: A Practical Approach J. Fungi. 2017, 3(2):19. doi:10.3390/jof3020019.

Aspiroz C, Sevil M, Toyas C, Gilaberte Y. Photodynamic Therapy With Methylene Blue for Skin Ulcers Infected With Pseudomonas aeruginosa and Fusarium spp. Actas Dermo-Sifiliográficas (English Edition). 2017, 108(6): e45-e48. doi: 10.1016/j.ad.2016.11.020.

Bain BJ, Bates I, Laffan MA. Dacie and Lewis Practical Haematology. 2017, 12th edition, Elsevier, p.600. doi: 10.1016/C2014-0-01046-5

Baltazar LM, Ray A, Santos DA, Cisalpino PS, Friedman AJ and Nosanchuk JD. Antimicrobial photodynamic therapy: an effective alternative approach to control fungal infections. Front. Microbiol. 2015, 6:202. doi: 10.3389/fmicb.2015.00202.

Bhatti M, MacRobert A, Meghji S, Henderson B, Wilson M. A study of the uptake of toluidine blue O by Porphyromonas gingivalis and the mechanism of lethal photosensitization. Photochem. Photobiol. 1998, 68:370–376. doi: 10.1111/j.1751-1097.1998.tb09694.x.

Blanco LAA, Crispim JS, Fernandes KM, de Oliveira LL, Pereira MF, Bazzolli DMS, Martins GF. Differential cellular immune response of Galleria mellonella to Actinobacillus pleuropneumoniae. Cell Tissue Res. 2017, 370(1):153-168. doi: 10.1007/s00441-017-2653-5.

Bonifaz A, Saldaña M, Araiza, J, Mercadillo P, Tirado-Sánchez A. Two simultaneous mycetomas caused by *Fusarium verticillioides* and *Madurella mycetomatis.* Rev. Inst. Med. Trop. S. Paulo. 2017, 59:e55. doi: 10.1590/s1678-9946201759055

Chibebe Junior J, Sabino CP, Tan X, Junqueira JC, Wang T, Fuchs BB, Jorge AOC, Tegos GP, Hamblin MR, Mylonakis E. Selective photoinactivation of *Candida albicans* in the non-vertebrate host infection model *Galleria mellonella* BMC Microbiology. 2013, 13:217. doi: 10.1186/1471-2180-13-217.

Clinical and Laboratory Standards Institute, 2008. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi, 2nd ed. Approved standard M38-A. Clinical and Laboratory Standards Institute, Wayne, PA.

Coleman JJ, Muhammed M, Kasperkovitz PV, Vyas JM, and Mylonakis E. *Fusarium* pathogenesis investigated using *Galleria mellonella* as a heterologous host. Fungal Biol. 2011, 115(12):1279–1289. doi:10.1016/j.funbio.2011.09.005.

Dai T, de Arce VJB, Tegos GP, Hamblin MR. Blue Dye and Red Light, a Dynamic Combination for Prophylaxis and Treatment of Cutaneous *Candida albicans* Infections in Mice. Antimicrobial Agents And Chemotherapy, 2011, 55(12):5710–5717. doi:10.1128/AAC.05404-11.

Dai T, Fuchs BB, Coleman JJ, Prates RA, Astrakas C, St.Denis TG, Ribeiro MS, Mylonakis E, Hamblin MR, Tegos GP. Concepts and principles of photodynamic

therapy as an alternative antifungal discovery platform. Frontiers in Microbiology, 2012, 3(120). doi: 10.3389/fmicb.2012.00120.

de Menezes HD, Rodrigues GB, Teixeira SDP, Massola NS, Bachmann L, Wainwright M, Braga GUL. In Vitro Photodynamic Inactivation of Plant-Pathogenic Fungi Colletotrichum acutatum and Colletotrichum gloeosporioides with Novel Phenothiazinium Photosensitizers. Applied and Environmental Microbiology. 2014, 80(5): 1623–1632. doi: 10.1128/AEM.02788-13.

de Menezes HD, Tonani L, Bachmann L, Wainwright M, Braga GUL, von Zeska Kress MR. Photodynamic treatment with phenothiazinium photosensitizers kills both ungerminated and germinated microconidia of the pathogenic fungi Fusarium oxysporum, Fusarium moniliforme and Fusarium solani. Journal of Photochemistry and Photobiology B: Biology, 2016, 164: 1–12. doi: 10.1016/j.jphotobiol. 2016.09.008.

Freire F, Ferraresi C, Jorge AOC, Hamblin MR. Photodynamic therapy of oral Candida infection in a mouse model. Journal of Photochemistry & Photobiology, B: Biology. 2016, 159: 161–168. Doi: 10.1016/j.jphotobiol.2016.03.049.

Gad F, Zahra T, Hasan T, Hamblin MR. Effects of growth phase and extracellular slime on photodynamic inactivation of gram-positive pathogenic bacteria. Antimicrob. Agents Chemother. 2004, 48:2173–2178. doi: 10.1128/AAC.48.6.2173-2178.2004.

Giannouli M, Palatucci AT, Rubino V, Ruggiero G, Romano M, Triassi M, Ricci V, Zarrilli R. Use of larvae of the wax moth Galleria mellonella as an in vivo model to study the virulence of Helicobacter pylori. BMC Microbiology, 2014, 14:228. doi.org/10.1186/s12866-014-0228-0.

Guilhermetti E, Takahachi G, Shinobu CS, and Svidzinski TIE. Fusarium spp. as agents of onychomycosis in immunocompetent hosts. International Journal of Dermatology 2007, 46: 822–826. doi: 10.1111/j.1365-4632.2007.03120.x.

Hamblin MR and Hasan T. Photodynamic therapy: a new antimicrobial approach to infectious disease? Photochem Photobiol Sci. 2004, 3(5):436–450. doi:10.1039/b311900a.

Homa M, Shobana CS, Singh YRB, Manikandan P, Selvam KP, Kredics L, Narendran V, Vágvölgyi C and Galgoczy L. *Fusarium* keratitis in South India: causative agents, their antifungal susceptibilities and a rapid identification method for the *Fusarium solani* species complex. Mycoses, 2013, 56: 501–511 doi:10.1111/myc.12062.

Katkar VJ, Tankhiwale SS, Kurhade A. *Fusarium solani* mycetoma. Indian J Dermatol. 2011, 56(3): 315–317. doi: 10.4103/0019-5154.82490.

Kelly J and Kavanagh K. Caspofungin primes the immune response of the larvae of Galleria mellonella and induces a non-specific antimicrobial response. Journal of Medical Microbiology. 2011, 60:189–196. doi: 10.1099/jmm.0.025494-0.

Kubin A, Wierrani F, Jindra RH, Loew HG, Grunberger W, Ebermann R, Alth G. Antagonistic effects of combination photosensitization by hypericin, meso-tetrahydroxyphenylchlorin (mTHPC) and photofrin II on Staphylococcus aureus. Drugs Exp. Clin. Res. 1999, 25:13–21.

Lionakis MS and Kontoyiannis DP. Drosophila melanogaster as a model organism for invasive aspergillosis. Methods Mol Biol. 2012, 845:455–468. doi:10.1007/978-1-61779-539-8_32.

Nucci M and Anaissie E. Fusarium Infections in Immunocompromised Patients. Clinical microbiology reviews. 2007, 20(4):695–704. doi:10.1128/CMR.00014-07.

Rodrigues GB, Ferreira LKS, Wainwright M, Braga GUL. Susceptibilities of the dermatophytes Trichophyton mentagrophytes and T. rubrum microconidia to photodynamic antimicrobial chemotherapy with novel phenothiazinium photosensitizers and red light. Journal of Photochemistry and Photobiology B: Biology. 2012, 116: 89–94. doi: 10.1016/j.jphotobiol.2012.08.010.

Rosseti IB, Chagas LR, Costa MS. Photodynamic antimicrobial chemotherapy (PACT) inhibits biofilm formation by Candida albicans, increasing both ROS production and membrane permeability. Lasers Med Sci. 2014, 29(3):1059-64. doi: 10.1007/s10103-013-1473-4.

Santos JD, Alvarenga JA, Rossoni RD, García MT, Moraes RM, Anbinder AL, Jorge AOC, Junqueira JC. Immunomodulatory effect of photodynamic therapy in Galleria mellonella infected with Porphyromonas gingivalis Microbial Pathogenesis. 2017, 110:507e511. doi: 10.1016/j.micpath.2017.07.045.

Schafer M, Schmitz C, Facius R, Horneck G, Milow B, Funken KH, Ortner J. Systematic study of parameters influencing the action of Rose Bengal with visible light on bacterial cells: comparison between the biological effect and singlet-oxygen production. Photochem. Photobiol. 2000, 71: 514–523. doi: 10.1562/0031-8655(2000)0710514SSOPIT2.0.CO2.

Tsai CJY, Loh JMS, Proft T. Galleria mellonella infection models for the study of bacterial diseases and for antimicrobial drug testing. Virulence, 2016, 7(3): 214–229. doi: 10.1080/21505594.2015.1135289.

Tonani L, Morosini NS, de Menezes HD, Bonifacio da Silva MEN, Wainwright M, Braga GUL, von Zeska Kress MR. In vitro susceptibilities of Neoscytalidium spp. sequence types to antifungal agents and antimicrobial photodynamic treatment with phenothiazinium photosensitizers. Fungal Biol. 2018, 122(6):436-448. doi: 10.1016/j.funbio.2017.08.009.

Wainwright M, Phoenix DA, Laycock SL, Wareing DR, Wright PA. Photobactericidal activity of phenothiazinium dyes against methicillin-resistant strains of Staphylococcus aureus. FEMS Microbiol. Lett. 1998, 160:177–181. doi: 10.1111/j.1574-6968.1998.tb12908.x

Wainwright M, Crossley KB. Methylene blue – a therapeutic dye for all seasons? J. Chemother. 2002, 14:431-443. doi: 10.1179/joc.2002.14.5.43.

Wainwright M. The development of phenothiazinium photosensitisers. Photodiagnosis and Photodynamic Therapy. 2005, 2, 263-272. doi:10.1016/S1572-1000(05)00110-9.

Wainwright, M. Photosensitizers in biomedicine. John Wiley & Sons, Ltd., 1st edition, 2009, ISBN 978-0-470-51060-5, 269 p.

Wainwright M, Meegan K, Loughran C. Phenothiazine Photosensitisers IX. Tetra- and pentacyclic derivatives as photoantimicrobial agents. Dyes Pigm. 2011, 91:1-5. doi: 10.1016/j.dyepig.2011.02.001.

Wainwright M, Smalley H, Scully O, Lotfipour E. Comparative photodynamic evaluation of new phenothiazinium derivatives against Propionibacterium acnes. Photochem. Photobiol. 2012, 88:523-526. doi: 10.1111/j.1751-1097.2011.01021.x.

Wainwright M, Maisch T, Nonell S, Plaetzer K, Almeida A, Tegos GP, Hamblin MR. Photoantimicrobials-are we afraid of the light? Lancet Infect Dis. 2017, 17(2):e49-e55. doi: 10.1016/S1473-3099(16)30268-7.

Wainwright M, Antczak J, Baca M, LoughranC , MeeganK. Phenothiazinium photoantimicrobials with basic side chains. Journal of Photochemistry and Photobiology B: Biology. 2015, 150:38–43. doi: 10.1016/j.jphotobiol.2014.12.017.

Wainwright M. Synthetic, small-molecule photoantimicrobials - a realistic approach. Photochem Photobiol Sci. 2018. doi: 10.1039/c8pp00145f.

Zhou S, Sun Z, Ye Z, Wang Y, Wang L, Xing L, Qiu H, Huang N, Luo Y, Zhao Y, Gu Y. *In vitro* photodynamic inactivation effects of benzylidene cyclopentanone photosensitizers on clinical fluconazole-resistant *Candida albicans*. Photodiagnosis and Photodynamic Therapy. 2018, 22: 178–186. doi: 10.1016/j.pdpdt.2018.04.001.

- TABLE -

Table 01 – Susceptibility of *F. keratoplasticum* and *F. moniliforme* to commercial antifungal drugs.

	VOR	ITR	POS	AMB
	MIC (μg mL^{-1})			
***F. keratoplasticum* (ATCC®36031™)**	8	64	> 64	1
***F. moniliforme* (ATCC®38159™)**	4	32	> 64	2
***Aspergillus flavus* (ATCC®204304™)**	0.5	0.25	0.25	1

VOR, voriconazole; ITR, itraconazole; POS, posaconazole; AMB, amphotericin B; MIC, minimal inhibitory concentration.

- FIGURES -

Figure 1: Virulence of *F. keratoplasticum* and *F. moniliforme* in *G. mellonella* larvae. Larvae were infected with microconidia of *F. keratoplasticum* and *F. moniliforme* at the concentration of 1×10^1 to 1×10^5 microconidia/larva. Ten larvae were used in each condition and the survival was accompanied daily up to 9 days. PBS, phosphate buffer saline.

Figure 2: Toxicity of phenothiazinium photosensitizers in *G. mellonella* larvae. Larvae were inoculated with MB, NMBN and S137. Ten larvae were used in each condition and the survival was accompanied daily up to 10 days. MB, methylene blue; NMBN, new methylene blue N; S137, pentacyclic MB derivative; PBS, phosphate buffer saline; light, 15 J cm^{-2}; dark, light absence; PS, photosensitizer; 1×, 750 μM (MB) and 100 μM (NMBN and S137); 2×, 1500 μM (MB) and 200 μM (NMBN and S137); 3×, 3000 μM (MB) and 400 μM (NMBN and S137).

Figure 3: APDT in *G. mellonella* larvae infected with *F. keratoplasticum*. Larvae were infected with microconidia of *F. keratoplasticum* and submitted to APDT with MB, NMBN and S137. Ten larvae were used in each condition and the survival was

accompanied daily up to 10 days. MB, methylene blue; NMBN, new methylene blue N; S137, pentacyclic MB derivative; PBS, phosphate buffer saline; L-, dark; L+, 15 J cm^{-2}; *Fk*, *F. keratoplasticum*; PS, photosensitizer; 1×, MB (750 μM), NMBN and S137 (100 μM); 1×, 750 μM (MB) and 100 μM (NMBN and S137); 2×, 1500 μM (MB) and 200 μM (NMBN and S137).

Figure 4: APDT in *G. mellonella* larvae infected with *F. moniliforme*. Larvae were infected with microconidia of *F. moniliforme* and submitted to APDT with MB, NMBN and S137. Ten larvae were used in each condition and the survival was accompanied daily up to 10 days. MB, methylene blue; NMBN, new methylene blue N; S137, pentacyclic MB derivative; PBS, phosphate buffer saline; L-, dark; L+, 15 J cm^{-2}; *Fm*, *F. moniliforme*; PS, photosensitizer; 1×, 750 μM (MB) and 100 μM (NMBN and S137); 2×, 1500 μM (MB) and 200 μM (NMBN and S137).

Figure 5: Fungal burden in the hemolymph of *G. mellonella* after APDT with MB, NMBN and S137. Larvae were infected with microconidia of *F. keratoplasticum* and *F. moniliforme* and submitted to APDT with MB (1500 μM), NMBN (200 μM) and S137 (200 μM). After 30 min the hemolymph was collected. CFU are presented as the mean and standard deviation of three larvae. *$P < 0.05$; CFU, colony forming units; MB, methylene blue; NMBN, new methylene blue N; S137, pentacyclic MB derivative; L-, dark; L+, 15 J cm^{-2}.

Figure 6 : Total Hemocyte Count (THC) in the hemolymph of *G. mellonella* larvae non infected and infected with *F. keratoplasticum* and *F. moniliforme*. Larvae were non infected and infected with microconidia of *F. keratoplasticum* and *F. moniliforme,* and submitted to APDT with MB (1500 μM), NMBN (200 μM) and S137 (200 μM). After 30 min the hemolymph was collected. THC are presented as the mean and standard deviation of three larvae. Naïve, control larvae without inoculum; *$P < 0.05$; PBS, phosphate buffer saline; MB, methylene blue; NMBN, new methylene blue N; S137, pentacyclic MB derivative; L-, dark; L+, 15 J cm^{-2}.