

Antimicrobial photodynamic therapy with phenothiazinium photosensitizers in non-vertebrate model *Galleria mellonella* infected with *Fusarium keratoplasticum* and *Fusarium moniliforme*

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ABSTRACT

Fusarium keratoplasticum and *Fusarium moniliforme* are filamentous fungi common in the environment and cause mycosis in both animals and plants. Human infections include mycetoma, 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.

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 [1,2]. 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 [1,3].

The PSs initially proposed for antimicrobial use were phenothiazinium dyes [4–6], perylenequinones [7] and rose bengal [8]. 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) [1,9]. Despite the enormous existing confidence in commercial PS, the study of new molecules, including new derivatives of MB, offers great improvements in antimicrobial use [10–13]. New methylene blue (NMBN) is a close derivative of MB and was initially used for the routine staining of reticulocytes [14]. NMBN is an active photo-antimicrobial *in vitro* against *Trichophyton* sp. [15], and *Candida* species [15], and is a viable approach for prophylaxis and treatment of cutaneous infections caused by *C. albicans* [16]. S137, also named DO15 (1,11-die-thyl-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

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phenothiazinium derivative of MB based on suitable tetrahydroquinolines [17,18]. This MB derivative was studied *in vitro* for photoinactivation of bacteria and fungi. S137 is effective against the Gram-positive bacterium *Propionibacterium acnes* [19], and fungal species of the genera *Trichophyton* [15], *Candida* [15], *Colletotrichum* [20], *Fusarium* [21], and *Neoscytalidium* [22].

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 number of immunocompromised patients and improved identification in the clinical laboratory [23]. The most common clinically-important species is *Fusarium solani* species complex (e.g., *F. fal-ciforme*, *F. keratoplasticum* and *F. petrophilum*), followed by *F. oxysporum*, *F. moniliforme* (synonym *F. verticillioides* (Sacc.) Nirenberg), *F. dimerum*, and *F. proliferatum* [23]. Mycosis in humans, such as mycetoma [24,25], keratitis [26] and onychomycosis [27] 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 [28]. Difficulties in treatment are attributed to primary (intrinsic) and secondary resistance profiles of *Fusarium* spp. to currently used antifungals [23,29].

The antimicrobial properties of drugs have been studied by employing insect models of microbial infections such as the greater wax moth *Galleria mellonella* [30]. *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 [31,32]. 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 [33].

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 28 °C 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) [34]. 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 × 10⁴ microconidia mL⁻¹. After 48 h of incubation at 28 °C, 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 [18]. 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 [21]. The fluence of 15 J cm⁻² was achieved after 19.5 min of light exposure.

2.4. *Galleria mellonella* as a 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*, the 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 10⁴ microconidia of *Fusarium* spp. into the hemocoel of each larva 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 10¹, 10², 10³, 10⁴ and 10⁵ microconidia of *F. keratoplasticum* and *F. moniliforme* were tested in *G. mellonella* larvae with 5 µL injection of 2 × 10³, 2 × 10⁴, 2 × 10⁵, 2 × 10⁶ and 2 × 10⁷ 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* was tested based on minimum fungicidal concentrations (MFC) determined by De Menezes et al. [21]. 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. keratoplacticum* and *F. moniliforme*. The final concentration of microconidia injected in each larva was 10^4 microconidia ($5 \mu\text{l}$ 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 microconidia suspension was prepared in PBS or in the PS solution in which both microconidia and PS were injected together in $5 \mu\text{l}$ 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 min 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 $\mu\text{g mL}^{-1}$ chloramphenicol. The numbers of colony-forming units (CFU) were counted after 48 h of incubation at 30°C . 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. [35]. 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 a hemocytometer and observed under a light microscope (Coleman with $40\times$ objective). The experiments were repeated twice and representative experiments are presented.

3. Results

3.1. Antifungal susceptibility test

The antifungal susceptibility of *F. keratoplacticum* 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 [34]. The analysis revealed that itraconazole and posaconazole exhibited *in vitro* activity against *F. keratoplacticum* and *F. moniliforme* (MIC value 32 to $> 64 \mu\text{g mL}^{-1}$). Voriconazole and amphotericin B were active with MIC ranges of $4\text{--}8 \mu\text{g mL}^{-1}$ and $1\text{--}2 \mu\text{g mL}^{-1}$, respectively (Table 1). Therefore, the high MIC values of itraconazole and posaconazole required to inactivate *F. keratoplacticum* and *F. moniliforme* indicate that these strains are resistant to these antifungal agents.

Table 1

Susceptibility of *F. keratoplacticum* and *F. moniliforme* to commercial antifungal drugs.

	VOR MIC ($\mu\text{g mL}^{-1}$)	ITR MIC ($\mu\text{g mL}^{-1}$)	POS	AMB
<i>F. keratoplacticum</i> (ATCC [®] 36031 [™])	8	64	> 64	1
<i>F. moniliforme</i> (ATCC [®] 38159 [™])	4	32	> 64	2
<i>Aspergillus flavus</i> (ATCC [®] 204304 [™])	0.5	0.25	0.25	1

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

3.2. Infective inoculum effect of *F. keratoplacticum* 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. keratoplacticum* and *F. moniliforme* against *G. mellonella* (Fig. 1). Inocula of 10^1 , 10^2 and 10^3 microconidia/larva of *F. keratoplacticum* 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. keratoplacticum* 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 microconidia inoculum in a dose-dependent manner during 9 days incubation (Fig. 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. keratoplacticum* killed 100% of the larvae at 8 days post-infection ($P \leq 0.05$ - Fig. 1). The inoculum of 10^4 microconidia/larva was considered optimum for the production of acute infection with *F. keratoplacticum* 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 (10–20%) 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 (Fig. 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. keratoplacticum* 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. keratoplacticum* and *F. moniliforme*. Both species were able to kill 100% of the larvae, respectively, by the 5th and 7th days, respectively, after microconidial injection (Fig. 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. keratoplacticum* and *F. moniliforme* (Figs. 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. keratoplacticum* 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. keratoplacticum* 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) (Fig. 3 and 4). APDT with S137 was the most effective for both fungal species.

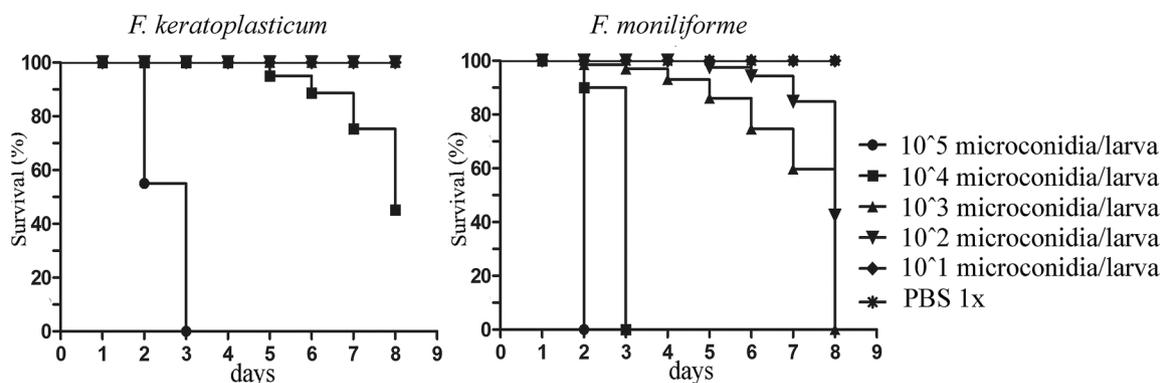


Fig. 1. Virulence of *F. keratoplacticum* and *F. moniliforme* in *G. mellonella* larvae. Larvae were infected with microconidia of *F. keratoplacticum* and *F. moniliforme* at the concentration of 1×10^1 to 1×10^5 microconidia/larva. Ten larvae were used in each condition and the survival was accompanied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

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

F. keratoplacticum 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. keratoplacticum* and *F. moniliforme* in infected larvae. Conversely, APDT with MB, NMBN and S137 in *G. mellonella*-infected larvae reduced the survival of *F. keratoplacticum* microconidia to 38, 57 and 2%, respectively. The survival levels of *F. moniliforme* microconidia in infected-larvae were 69, 37 and 21%, respectively (Fig. 5). This indicates that APDT with MB, NMBN and S137 in *G. mellonella*-infected larvae are able to significantly reduce the viability of *F. keratoplacticum* 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. keratoplacticum* 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 (Fig. 6). Larvae infected with *F. keratoplacticum* and *F. moniliforme* had THCs of 159/ μL and 48/ μL , respectively. Once the larva was infected with microconidia of *F. keratoplacticum* 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 (Fig. 6). The only exception was the decrease of THC in the APDT (15 J cm^{-2}) with NMBN in larvae

infected with *F. keratoplacticum* (figure 06). Regardless of the THC decreasing, the larval survival remained high after APDT (Fig. 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. keratoplacticum*, demonstrating that the cellular 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.

4. 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. keratoplacticum* and *F. moniliforme*.

First, we determined the virulence of *F. keratoplacticum* 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. keratoplacticum*, 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 [36]. 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*) [36]. 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

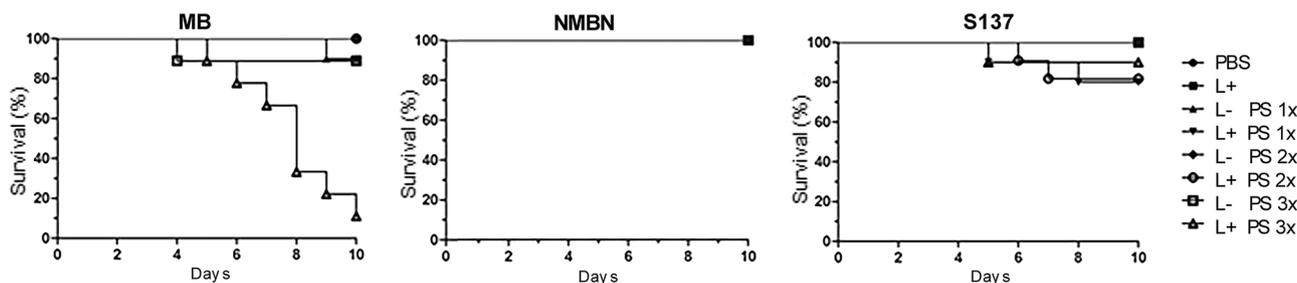


Fig. 2. Toxicity of phenothiazinium photosensitizers in *G. mellonella* larvae. Larvae were inoculated with MB, NMBN and S137. Ten larvae were used in each condition and the survival was accompanied daily up to 10 days. MB, methylene blue; NMBN, new methylene blue N; S137, pentacyclic MB derivative;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

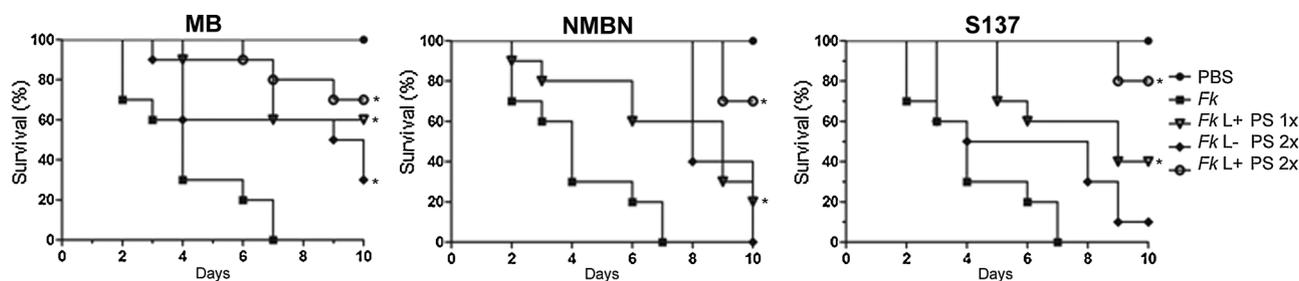


Fig. 3. APDT in *G. mellonella* larvae infected with *F. keratoplaticum*. Larvae were infected with microconidia of *F. keratoplaticum* and submitted to APDT with MB, NMBN and S137. Ten larvae were used in each condition and the survival was accompanied daily up to 10 days. MB, methylene blue; NMBN, new methylene blue N; S137, pentacyclic MB derivative; PBS, phosphate buffer saline; L-, dark; L+, 15 J cm⁻²; Fk, *F. keratoplaticum*; PS, photosensitizer; 1 ×, MB (750 μM), NMBN and S137 (100 μM); 2 ×, 1500 μM (MB) and 200 μM (NMBN and S137).

microconidial inoculum dose. Notwithstanding, we determined a lethal concentration of *F. moniliforme* and *F. keratoplaticum* microconidia against *G. mellonella* to study the effects of APDT in experimental infection assay.

Fusarium spp. are considered multi-resistant pathogens [37] and are resistant to most antifungal agents [28]. Our results demonstrate the resistance of *F. keratoplaticum* 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 [37]. The effect of APDT is the production of reactive oxygen species (ROS) which are highly reactive against fungal biomolecules [3]. Since many different fungal biomolecules are potentially damaged, the selection of resistant strains is greatly reduced, making APDT a 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. keratoplaticum* and *F. moniliforme*. This model of infection has the potential to facilitate the *in vivo* study of pathogen-host crosstalk [38]. We previously showed the *in vitro* efficacy of APDT with MB, NMBN and S137 against microconidia of *F. keratoplaticum* (*F. solani* species complex) and *F. moniliforme* [21]. 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. keratoplaticum* 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* [38] and the Gram-negative bacterium *Porphyromonas gingivalis* [39], and were able to prolong the survival of the infected larvae. MB and NMBN were effective in APDT of an immunosuppressed mouse model of oral candidiasis infection [40]. MB is often used since was the first antimicrobial PS clinically approved to human use [9]. Skin ulcers infected with *Fusarium* spp. were successfully treated with

MB and red light [41]. Therefore, APDT with MB and NMBN is effective in different hosts against different infecting microorganism, including azole-resistant strains of the filamentous fungi *F. keratoplaticum* and *F. moniliforme*.

The production of new, potent and clinically compatible PSs is of increasing interest [1,9]. We showed the *in vitro* effectiveness of the novel pentacyclic phenothiazinium PS S137 [21], 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. keratoplaticum* 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 [3,42]. 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. keratoplaticum* and *F. moniliforme* microconidia [21] and *Neoscytaldium* spp. arthroconidia [22]. Additionally, the *in vitro* killing effect was demonstrated [21], and is emphasized by the reduced microconidia viability reported here *in vivo* in *G. mellonella* larvae infected with *F. keratoplaticum* 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 [38]. Additionally, the production of ROS and consequent cell permeability is increased *in vitro* in *C. albicans* upon APDT with TB [42]. 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.

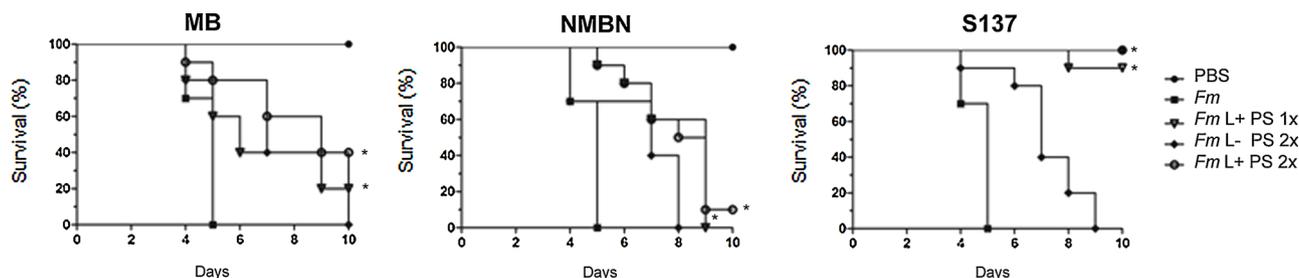


Fig. 4. APDT in *G. mellonella* larvae infected with *F. moniliforme*. Larvae were infected with microconidia of *F. moniliforme* and submitted to APDT with MB, NMBN and S137. Ten larvae were used in each condition and the survival was accompanied daily up to 10 days. MB, methylene blue; NMBN, new methylene 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).

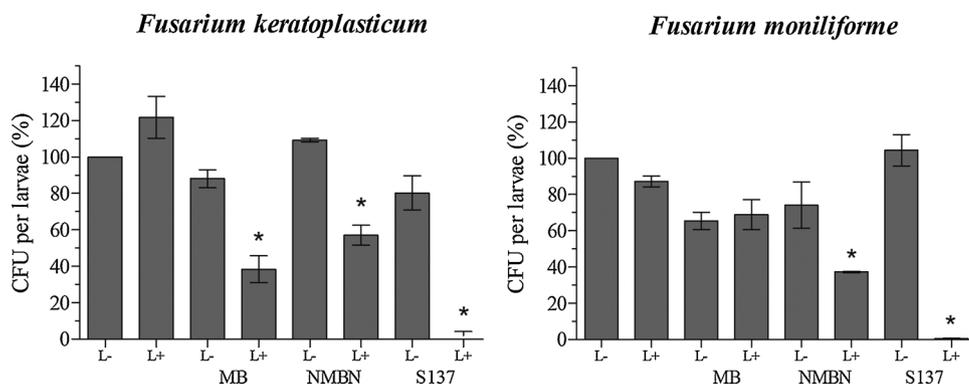


Fig. 5. Fungal burden in the hemolymph of *G. mellonella* after APDT with MB, NMBN and S137. Larvae were infected with microconidia of *F. keratoplasticum* and *F. moniliforme* and submitted to APDT with MB (1500 μM), NMBN (200 μM) and S137 (200 μM). After 30 min the hemolymph was collected. CFU are 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.

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 with low larval survival and increase in hemocyte count is correlated with 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* [39] and *C. albicans* [38]. 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.

5. 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 increased 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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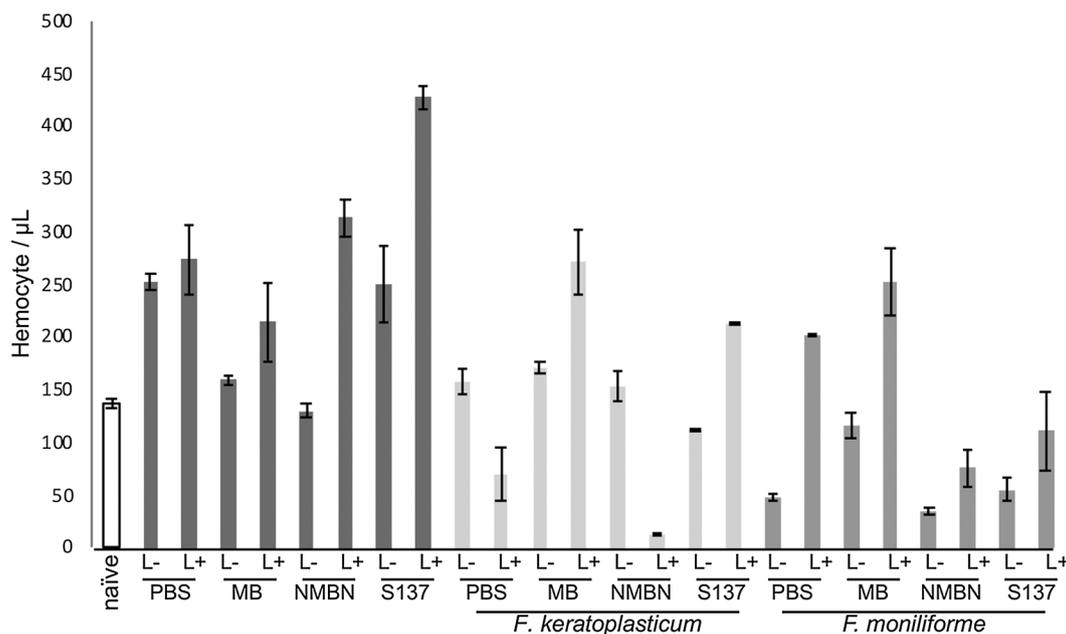


Fig. 6. Total Hemocyte Count (THC) in the hemolymph of *G. mellonella* larvae non-infected and infected with *F. keratoplasticum* and *F. moniliforme*. Larvae were non-infected and infected with microconidia of *F. keratoplasticum* and *F. moniliforme*, and submitted to APDT with MB (1500 μM), NMBN (200 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.

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