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ORIGINAL ARTICLE

The role of melanin in *Aspergillus* tolerance to biocides and photosensitizers

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Significance and Impact of the Study: Cationic photosensitizers such as toluidine blue O (TBO) are being investigated as low-cost and non-toxic alternatives to traditional biocides. Compared to bacteria, studies addressing their fungicidal properties are sparse. We observed profound differences in biocidal susceptibility between *Candida albicans* and *Aspergillus brasiliensis* conidia which was linked to melanin production. Our results demonstrate that cationic biocides, such as TBO and benzalkonium chloride, are poorly suited to applications where the control of melanin-producing fungi is required.

Keywords
Aspergillus, benzalkonium chloride, *Candida*, photosensitizer, resistance.

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Abstract

Cationic biocides are widely utilized for surface disinfection. Photosensitizers such as toluidine blue O (TBO) produce reactive oxygen species following light excitation and are being investigated as novel biocides for similar applications. *Aspergillus brasiliensis* conidia contain melanin which protects against environmental stressors. The negative charge and antioxidant properties of melanin may confer resistance to photosensitizers and other biocides. In this study, the yeasticidal and fungicidal activity benzalkonium chloride (BZC), sodium dichloroisocyanurate (NaDCC) and TBO with red light were examined using quantitative suspension tests. All three biocides were highly effective against *Candida albicans* and > 5·0 $\log_{10}$ reductions in viability were attainable within 5 minutes. Wild-type *A. brasiliensis* conidia were highly tolerant to treatment and 0·4 $\log_{10}$ reductions in viability were observed within the same time frame when treated with TBO or BZC. NaDCC was markedly more effective. Inhibition of melanin biosynthesis by culturing with 100 μg ml^{-1} kojic acid resulted in a hypopigmented phenotype with significantly increased sensitivity to all three biocides. These observations indicate that melanin is a significant contributor towards *A. brasiliensis* tolerance of biocides and photosensitizers and demonstrate that cationic biocides are poorly suited to applications where the control of *A. brasiliensis* is required.

Introduction

Photosensitizers are compounds which produce reactive oxygen species (ROS) following excitation by visible light. Excitation of the photosensitizer from a ground state yields a quantum transition to an excited triplet state, which in turn facilitates the conversion of triplet molecular oxygen into a more energetic singlet by spin inversion. Singlet oxygen has biocidal properties but is only created during light excitation and so photosensitizers have been proposed as a benign solution for the control of microorganisms in various environments (Luksienė *et al.* 2009).

Toluidine blue O (TBO) is a cationic phenothiazine photosensitizer which strongly absorbs 630 nm red light and produces singlet oxygen with a high quantum yield. Phenothiazines, which also include methylene blue, have been extensively researched as photoantimicrobials against a range of pathogenic species, including methicillin-resistant *Staphylococcus aureus* (Tseng *et al.* 2017), *Escherichia coli* (Kömerik and Wilson 2002), *Mycobacterium smegmatis* (Shim *et al.* 2016), enveloped (Chang *et al.* 1975) and non-enveloped viruses (Wong *et al.* 2010).

Fungi are major sources of contamination in pharmaceutical clean-rooms, food production plants and

healthcare surfaces (Weber *et al*. 2009; Vijayakumar *et al*. 2016; Snyder and Worobo 2018). The use of effective fungicides is essential for reducing the spread of mycoses and preventing costly recalls due to product contamination. TBO has a well-established safety profile and is licensed for in vivo medical use within the United Kingdom (Sweetman 2009) and so we seek to investigate its suitability as a non-toxic anti-fungal agent.

When compared to studies on prokaryotes, the fungicidal activity of photosensitizers is relatively overlooked. *Candida albicans*, an opportunistic pathogen which causes thrush and invasive bloodstream infections (Köhler *et al*. 2014), has been widely reported to be susceptible to the photodynamic inactivation by phenothiazines such as TBO and methylene blue (Souza *et al*. 2010; Pupo *et al*. 2011; Rodrigues *et al*. 2013). However, photoinactivation of *C. albicans* occurs approximately 10 times slower than in bacterial species such as *S. aureus* (Zeina *et al*. 2001). International testing standards such as EN 13624 state that fungicidal products should also be effective against the asexual reproductive spores (conidia) of *Aspergillus brasiliensis* (British Standards Institute 2013). *Aspergillus* conidia contribute to food spoilage in commercial environments and can cause severe respiratory infections in immunocompromised patients (Köhler *et al*. 2014). TBO and methylene blue have previously been observed to inactivate conidia of *Trichophyton* spp. (Rodrigues *et al*. 2012), *Aspergillus nidulans* and *Metarhizium anisopliae* (Gonzales *et al*. 2010). However, there is a lack of studies examining the efficacy of these photosensitizers against *A. brasiliensis* conidia.

Aspergillus brasiliensis belongs to *Aspergillus* section Nigri (i.e. black *Aspergillus*), which are strongly pigmentated by the presence of melanin in the cell wall. Melanin supports the cell wall structure and confers resistance to a range of environmental stressors (Pihet *et al*. 2009; Bayry *et al*. 2014). Fungal melanins are negatively charged (Nosanchuk and Casadevall 1997), interact strongly with cationic aromatic peptides (Nosanchuk *et al*. 1999) and have antioxidant properties against singlet oxygen and radical species (Hamilton and Holden 1999; Tada *et al*. 2010). Melanin extracted from *A. nidulans* has been observed to quench the oxidizing activity of biocides including sodium hypochlorite and hydrogen peroxide (Goncalves and Pombeiro-Sponchiado 2005). As melanin granules are arranged in concentric layers which may form a layer up to 200 nm thick in some species (Eisenman *et al*. 2005), we hypothesize that melanin may confer protection against biocides with complementary ionic charge by hindering their interaction with intracellular target sites.

Melanin production in *Aspergillus* spp. occurs through the dihydroxy naphthalene (DHN) and L-dihydroxy phenylalanine (L-DOPA) biosynthetic pathways (Langfelder *et al*. 2003). Most species predominantly utilize the DHN pathway, with *A. niger* and *A. flavus* being noted exceptions (Krijgsheld *et al*. 2013; Pal *et al*. 2014). Sub-inhibitory concentrations of antifungal compounds such as kojic acid and tricyclazole have been observed to inhibit melanin biosynthesis in *Gaeumannomyces* (Elliott 1995) and *Aspergillus* species (Pal *et al*. 2014). Tricyclazole has been stated to inhibit the biosynthesis of DHN-derived melanin, whilst kojic acid inhibits the L-DOPA pathway (Pal *et al*. 2014).

This study investigated the fungicidal properties of TBO and established the role of melanin in protection against photosensitization.

To further determine the role of melanin in protection against other biocides, susceptibility to the widely used quaternary ammonium compound benzalkonium chloride (BZC) and sodium dichloroisocyanurate (NaDCC) was also investigated. BZC does not produce ROS and its inclusion here was useful for determining the role of melanin charge in inhibition of biocidal activity. Ionic interactions between the biocides and melanin, as well as its antioxidant properties, were anticipated to play a key role in determining tolerance of *A. brasiliensis* conidia to such compounds.

Results and discussion

The three tested biocides were highly effective against suspensions of *Candida albicans* (Fig. 1a). Within 5 min of contact, ≥ 5 $\log_{10}$ reductions in yeast viability were observed for each in the presence of an interfering substance ($0{\cdot}3$ g l^{-1} BSA). As viability was decreased to below the lower limit of detection (4 CFU per ml) in all cases, there were no statistically significant differences in activity between biocides or at longer contact times ($P > 0{\cdot}05$; ANOVA). Neither red light alone nor the TBO formulation in the absence of light were biocidal, as $<0{\cdot}1$ $\log_{10}$ reductions were observed in suspensions treated under these conditions. This indicated that the combination of both treatments is required to affect microbial inactivation. As the observed reduction in viability was above the 4 $\log_{10}$ threshold required to substantiate claims of yeasticidal activity under the EN 13624 standard, a combination of TBO and red light may be suitable for applications in which the microbiological control of yeasts is required. Our observations corroborate those of previous studies (Souza *et al*. 2010; Pupo *et al*. 2011; Rodrigues *et al*. 2012).

In contrast to these observations, *Aspergillus brasiliensis* conidia were highly tolerant to biocide exposure. Over the 60-minute contact period, neither TBO nor BZC were able to achieve even a 1 $\log_{10}$ reduction in conidia

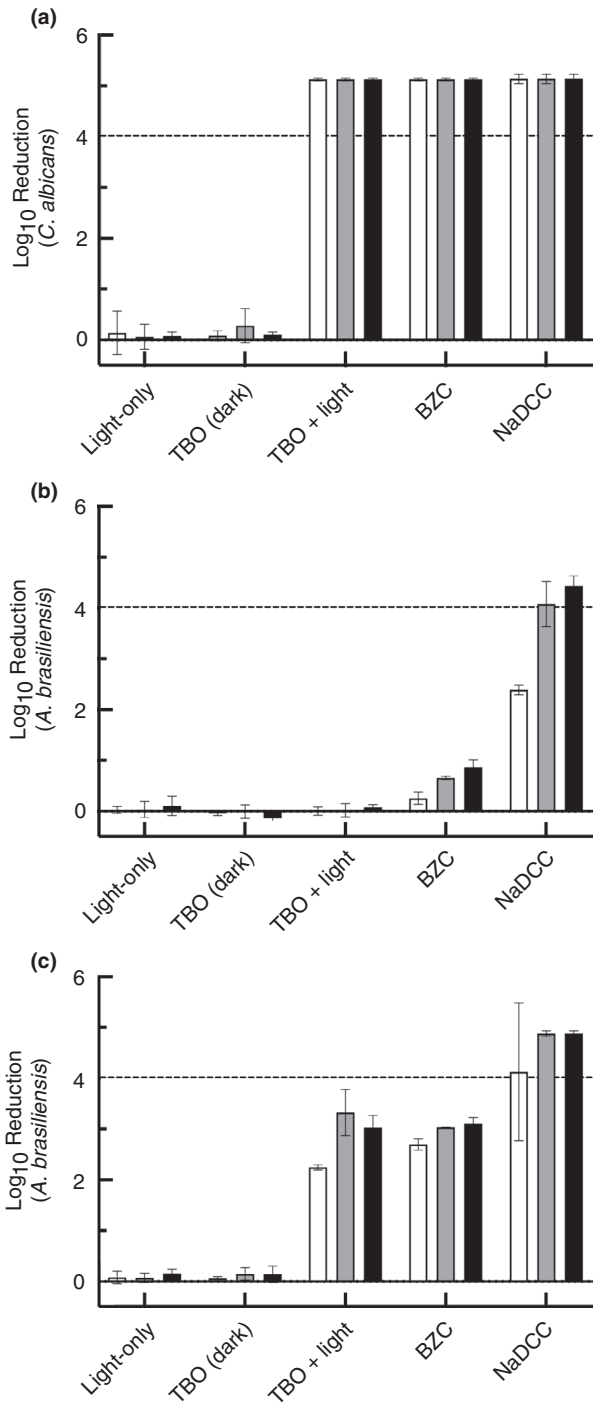

Figure 1 Biocidal activity of tested formulations against *Candida albicans* (a), *Aspergillus brasiliensis* conidia (b), and *A. brasiliensis* conidia propagated in the presence of 100 μg ml^{-1} kojic acid (c). Contact times: 5 (white), 15 (grey) and 60 (black) minutes. Dashed line denotes 4 $\log_{10}$ reduction threshold of efficacy. Light: 630 nm red light; TBO: 10 μmol l^{-1} toluidine blue O; BZC: 13·5 mmol l^{-1} benzalkonium chloride; NaDCC: 1 g^{-1} sodium dichloroisocyanurate. Error bars denote standard deviation.

viability (Fig. 1b). Differences in performance between the two biocides were not statistically significant ($P > 0{\cdot}05$; ANOVA, Tukey). In contrast, NaDCC was somewhat effective against conidia and a >4 $\log_{10}$ reduction in viability was attainable following 15 min of exposure.

The stark differences in observed efficacy between the two fungal species may be related to the abundance of melanin granules in the cell walls of *A. brasiliensis* conidia, which are absent in *C. albicans*. TBO and BZC are cationic compounds whose charges are complementary to melanin. Moreover, melanin has antioxidant properties and so may further protect conidia from ROS produced during photosensitization and through the oxidative species produced during NaDCC exposure.

Previous authors have reported that conidia of *Penicillium chrysogenum* (Gomes *et al.* 2011) and *Aspergillus flavus* (Temba *et al.* 2019) are susceptible to photosensitization on with cationic photosensitizers. However, these species are not considered to be part of the hyper-melanized section Nigri (Vesth *et al.* 2018). The susceptibility of these species to photosensitization may therefore be related to reduced melanin content. It is interesting to note that one group reported curcumin as an effective photosensitizer against conidia of *A. niger* (Al-Asmari *et al.* 2017), which is within section Nigri (Vesth *et al.* 2018). Curcumin is a naturally occurring photosensitizer which lacks an ionic charge (Glueck *et al.* 2017). This property makes it less likely to interact strongly with anionic cell wall melanins and so curcumin may better penetrate into areas of the conidia where melanin is absent, facilitating photodynamic destruction upon excitation.

Propagation of *A. brasiliensis* in the presence of the melanin biosynthesis inhibitors kojic acid and tricyclazole led to observable changes in colony phenotype (Fig. 2). Exposure to tricyclazole, which inhibits DHN-melanin biosynthesis, reduced the speed of maturation but did not inhibit melanin production. Exposure kojic acid, which inhibits the L-DOPA melanin pathway, resulted in colonies developing an off-white appearance. Microscopic examination of conidia harvested from these colonies indicated that exposure to kojic acid during propagation led to morphological changes. Non-inhibited conidia appeared dark and undulate, whilst those exposed to kojic acid were light and rounded. These observations suggest that the L-DOPA pathway is the dominant mechanism of melanin biosynthesis in *A. brasiliensis* and that exposure to 100 μg ml^{-1} kojic acid during propagation successfully inhibits melanin production in this species.

Inhibition of melanin biosynthesis had a profound impact on fungal susceptibility to TBO, BZC and NaDCC (Fig. 1c). Following a contact time of 5 min, viability

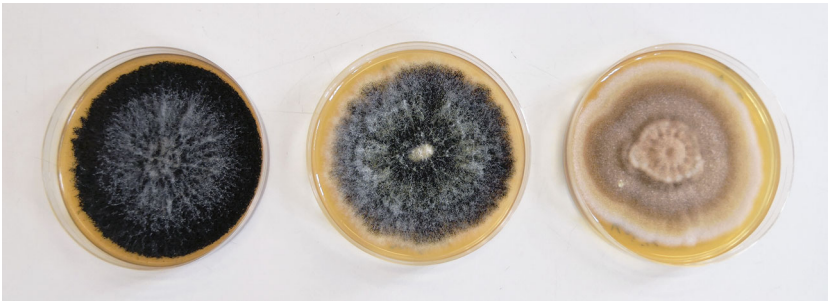

Figure 2 Colony pigmentation of *Aspergillus brasiliensis* propagated in absence (left) and presence (centre and right) of melanin biosynthesis inhibitors. Propagation with 100 μg ml^{-1} tricyclazole (centre) reduced the rate of maturation but resulted in a dark pigmented phenotype whilst 100 μg ml^{-1} kojic acid (right) led to a marked decrease in melanization.

decreased by 2·2 $\log_{10}$ for TBO and 2·7 $\log_{10}$ for BZC, demonstrating a significant change in susceptibility compared to non-inhibited controls ($P < 0{\cdot}01$; ANOVA, Tukey). In both cases, this further increased to >3$\log_{10}$ after 15 min, after which there was no additional decrease in viability following a 60-minute exposure. Differences in performance between the two biocides were not statistically significant at any of the time points tested ($P > 0{\cdot}05$; ANOVA, Tukey), indicating that TBO was of equal effectiveness to the widely used BZC against this phenotype. Biocidal performance of NaDCC against *A. brasiliensis* also increased markedly in melanin-inhibited conidia. At a 5-minute contact time, reductions were observed to increase from 2·4 to 4·1 $\log_{10}$ ($P < 0{\cdot}001$; ANOVA, Tukey).

In the case of TBO and BZC, neither biocide was able to reduce the viability of melanin-inhibited conidia to below the lower limit of detection (4 CFU per ml), even after prolonged exposure times of 60 min. This may be due to incomplete inhibition of melanin biosynthesis; colonies still retained a degree of pigmentation and were an off-white colour, rather than albino (Fig. 2). Propagation with a higher concentration of kojic acid may result in greater inhibition of melanin biosynthesis and improved biocide performance.

Our observations suggest that protection of conidia against TBO photosensitization arises as a function of both the antioxidant and anionic properties of melanin. Due to the high degree of tolerance observed against benzalkonium chloride, and previously described susceptibility towards curcumin (Al-Asmari *et al.* 2017), it is clear that the negative charge plays a key role in protection against cationic compounds. The increased susceptibility of melanized conidia to NaDCC clearly demonstrates that the antioxidant properties of melanin are also partly responsible for protection against oxidizing species, though were not sufficient to entirely protect conidia from the effects of NaDCC exposure. We propose that interactions between TBO and melanin lead to the photosensitizer becoming trapped within the outer cell wall layers, which contains a high density of melanin granules. Any ROS produced during subsequent photoexcitation would be localized to within this structure and would be rapidly quenched by proximal melanin, limiting damage to intracellular targets.

Overall, these data demonstrate that melanin is a significant contributor of *A. brasiliensis* tolerance to cationic photosensitizers and biocides, including TBO and the quaternary ammonium compound BZC. The anionic charge of this pigment is likely to create a barrier which prevents their interaction with target sites, thus attenuating their biocidal activity. Whilst exposure to kojic acid successfully sensitizes these spores by inhibiting melanin production, this approach is unlikely to be feasible in real-world surface disinfection applications as exposure to kojic acid must occur during fungal growth. Consequently, these agents are unsuitable for applications in which inactivation of such spores is required. As they share a similar mechanism of action to BZC, other surface-active cationic biocides may be less effective as fungicides than previously considered and so further investigation is necessary.

Whilst 1 g l^{-1} NaDCC was observed to be effective against *A. brasiliensis* in suspension tests, relatively long contact times of 15 min were required to achieve a satisfactory level of disinfection (i.e. 4 $\log_{10}$ reduction). Phase 2 validation tests such as EN 13697 more accurately model real-world use conditions and use a significantly smaller ratio of disinfectant to microbial challenge (British Standards Institute 2015). Slower performance would be anticipated to be observed in these tests unless biocide concentration was increased. Alternative biocides should be sought for applications where surface contamination with *A. brasiliensis* or other *Aspergillus* section Nigri species is suspected and where short contact times are required to reduce downtime. Within the field of

photosensitization, non-ionic photosensitizers such as curcumin may be a more promising source of anti-fungal agents.

Materials and methods

Biocides and diluent

Toluidine blue O and BZC were obtained from Sigma Aldrich and diluted in sterile 5 mmol l^{-1} sodium tetraborate (pH 9) to a concentration of 10 μmol l^{-1} (TBO) and 13·5 mmol l^{-1} (BZC). About 10 µmol l^{-1} TBO was selected for use as greater concentrations were observed to significantly attenuate light transmission through test suspensions in optimization experiments, reducing efficacy. About 13·5 mmol l^{-1} (0·5% w/v) BZC is considered a general use concentration for surface disinfection to ensure noncorrosivity and surface compatibility (Therapeutic Goods Administration 2017). A sodium dichloroisocyanurate (NaDCC)-based product certified as effective against *Aspergillus brasiliensis* (Chlor-Clean Tablets; Guest Medical) was selected as a positive control biocide and was prepared immediately before use by dissolving a single tablet in 1 l of sterile hard water (1·7 mmol l^{-1} $MgCl_2$; 3·3 mmol l^{-1} $CaCO_3$; 2·5 mmol l^{-1} $NaHCO_3$), as per manufacturer instructions; this provided a final concentration of 1 g l^{-1} NaDCC in the product test solution. To prevent aggregation of conidia during biocidal tests against *A. brasiliensis*, polysorbate-80 was added to each formulation to a concentration of 800 µmol l^{-1}.

Photosensitizer excitation source

An Eagle L330F/FW LED floodlight was used as an excitation source for the photosensitizer. This source emits red light at a peak wavelength of 630 nm and provides a luminous flux of 0·3 J/second at a distance of 55 mm.

Propagation of fungal species

Due to their inclusion within the EN 13624 fungicidal testing protocol, *Candida albicans* and *Aspergillus brasiliensis* were chosen as test organisms and were propagated on malt extract agar (MEA).

Spread plates of *Candida albicans* ATCC 10231 were incubated at 30°C for 48 h. Loopfuls of colonies were then transferred from the plates to 50 ml centrifuge tubes containing 10 ml of sterile tryptone sodium chloride (TSC) and 5 g of sterile glass beads. The suspensions were then vortexed for 1 min to separate aggregates and adjusted to an A_{630} of 1·0, yielding a cell density of 5–6 × 10^7 CFU per ml.

Conidia of *Aspergillus brasiliensis* NCTC 16404 were prepared by adding a 20 μl drop of freezer stock to the centre of an MEA plate. To inhibit melanin biosynthesis, additional plates containing 100 μg ml^{-1} kojic acid or tricyclazole were also prepared (Pal *et al.* 2014). Following inoculation, plates were incubated at 30°C for 12 days. Plates were photographed to record the appearance of colonies. About 100 μg ml^{-1} kojic acid was suitably effective for inhibiting melanin production, though it was observed that *A. brasiliensis* was still capable of growing on MEA containing up to 1600 μg ml^{-1} kojic acid in optimization experiments.

Conidia were harvested by adding 5 ml sterile TSC containing 800 µmmol^{-1} polysorbate-80 to each plate and scraping repeatedly with an inoculation loop. The liquid was aspirated from plates and transferred to a Büchner flask through a glass fritted filter under negative pressure to remove hyphae. Suspensions were examined by phase contrast microscopy to observe the conidia and confirm that hyphae were removed during filtration; micrographs were recorded. Filtrates prepared by this method contained 1–3 × 10^5 CFU per ml of conidia.

Determination of biocidal activity

Biocidal activity was determined using an approach based on suspension tests described by the EN 13624 standard (British Standards Institute 2013). Modifications to the test protocol were necessary to facilitate activation of the photosensitizer by the light source and to account for the reduced number of conidia able to be harvested from inhibited *Aspergillus* colonies.

In each test, 800 μl aliquot of biocide was added to a 1·5 ml microcentrifuge tube. About 100 μl of filter-sterilized BSA was then added as an interfering substance at a final concentration of 0·3 g l^{-1}, followed by 100 μl of fungal suspension (1–3 × 10^5 CFU per ml). For TBO-treated groups, 960 μl of test suspensions were transferred to sterile 35 mm diameter tissue culture dishes, yielding a 1-mm-thick suspension. Dishes were placed 55 mm directly beneath the light source and illuminated with 630 nm light for 5, 15 and 60 min. This was equivalent to a total light flux of 180, 540 and 2160 J respectively.

At completion of biocide exposure, 100 μl of suspension was transferred to 900 μl of neutralizer (30 g l^{-1} polysorbate-80; 30 g l^{-1} saponin; 3 g l^{-1} sodium thiosulphate; 3 g l^{-1} L-α-lethicin; 1 g l^{-1} L-histidine) and incubated for 5 min. The neutralized suspension was then serially diluted in sterile TSC, which was supplemented with 800 µmol^{-1} polysorbate-80 for *Aspergillus* samples. Spread plates were prepared by adding 250 μl of each dilution to an MEA plate and spreading with a sterile L-shaped spreader. Plates were incubated for 48 h at 30°C,

after which colonies were counted. Biocidal activity was calculated as $\log_{10}$ reduction in CFUs recovered from treated groups compared to untreated controls. Additional control tests were also performed to verify that observed changes in biocidal activity were not due to changes in susceptibility to red light or from TBO in the absence of light.

Statistical analysis

Statistical analyses were performed using Prism 8.1.0. Two-way analysis of variance (ANOVA) and Tukey's multiple comparison tests were used to determine whether changes in biocidal activity were statistically significant across time points and under various conditions of melanin inhibition. For presentation purposes, results for each species and phenotype were rendered into separate graphs after statistical analysis.

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Conflict of Interest

No conflict of interest declared.

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