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ORIGINAL ARTICLE

Antifungal efficacy of photodynamic therapy with TONS 504 for pathogenic filamentous fungi

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Abstract

The pathogenic filamentous fungi *Fusarium solani* (*F. solani*) and *Aspergillus fumigatus* (*A. fumigatus*) are common causes of fungal keratitis. We have here evaluated the antifungal efficacy of photodynamic antimicrobial chemotherapy (PACT) with the novel chlorin derivative TONS 504 and a light-emitting diode (LED) with a wavelength of 660 nm for these fungal species. Isolated fungal spores were irradiated at LED energies of 10, 20, or 30 J/cm^2 in the presence of TONS 504 at concentrations of 1 or 10 mg/L. As a control, spores were exposed to TONS 504 or LED radiation alone. The treated spores were then cultured on potato dextrose agar plates at 25 °C for 3 to 4 days before determination of colony formation as a measure of viability. Fungal growth was inhibited in a manner dependent on both LED energy and TONS 504 concentration. The inhibitory effect on *F. solani* was complete with TONS 504 at a concentration of 1 mg/L and LED irradiation at 30 J/cm^2 as well as at a TONS 504 concentration of 10 mg/L and LED irradiation at 10, 20, or 30 J/cm^2. In contrast, that on *A. fumigatus* was only partial at a TONS 504 concentration of 10 mg/L and LED irradiation at 20 or 30 J/cm^2. The antifungal effect of PACT on *A. fumigatus* was thus inferior to that on *F. solani*. PACT with TONS 504 and an LED thus warrants further evaluation with regard to its potential effectiveness for the treatment of infectious fungal keratitis.

Keywords Photodynamic antimicrobial chemotherapy (PACT) · Filamentous fungi · *Fusarium solani* · *Aspergillus fumigatus* · Fungal keratitis · Chlorin derivative

Introduction

Infectious keratitis can result in corneal perforation or scarring and is one of the major causes of blindness worldwide [1]. In the case of fungal keratitis, in which the most frequent causative agents are *Fusarium* and *Aspergillus* species, currently available antifungal drugs are not sufficiently effective, with new modes of treatment being urgently required.

Photodynamic therapy (PDT) has been developed to destroy cancer cells or to induce regression of new blood vessels through the generation of reactive oxygen species that results from the interaction of irradiating light of a specific wavelength with a photosensitizer accumulated in the target cells [2, 3]. This approach has thus been applied to the treatment of various types of cancer [2] and of age-related macular degeneration [4]. PDT dates back to the observation in 1900 that exposure of paramecia to sunlight in the presence of an acridine dye was cytotoxic [5]. The application of PDT to microorganisms declined, however, after the introduction of antibiotics, with the focus of this approach switching to cancer [6, 7]. The recent appearance of drug-resistant bacteria [8–10] has led to a resurgence of interest in the antimicrobial action of PDT, or photodynamic antimicrobial chemotherapy (PACT) as it has come to be known [11].

With the aim of developing new treatments for infectious keratitis, we recently showed that PACT with a newly developed chlorin derivative, TONS 504, as the photosensitizer and a light-

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emitting diode (LED) with a wavelength of 660 nm as the light source was effective for the elimination of methicillin-sensitive or methicillin-resistant *Staphylococcus aureus* as well as for that of *Pseudomonas aeruginosa* and acyclovir-sensitive or acyclovir-resistant herpes simplex virus type 1 in vitro [12–14]. We have now investigated the efficacy of PACT with TONS 504 for elimination of the pathogenic filamentous fungi *Fusarium solani* and *Aspergillus fumigatus*.

Materials and methods

Microorganisms

Strains of *F. solani* (NBRC 104627) and *A. fumigatus* (NBRC 8866) obtained from NITE Biological Resource Center were grown on potato dextrose agar (PDA) (Nissui Pharmaceutical, Tokyo, Japan) in slant tubes placed in an incubator at 25 °C. The fungal spores were isolated with the use of 0.05% polyoxyethylene (20) sorbitan monooleate (Wako, Osaka, Japan), a nonionic surfactant, and filtration through gauze. The resulting spore suspension was diluted with phosphate-buffered saline (PBS) for adjustment of colony-forming units (CFU).

Photosensitizer

The hydrophilic and cationic chlorin derivative TONS 504 [13,17-bis(1-carboxyethyl)carbamoyl(3-methylpyridine)-3-(1,3-dioxane-2-yl)methylidene-8-ethenyl-2-hydroxy-2,7,12,18-tetramethyl chlorin, diN-methyl iodide ($C_{51}H_{58}N_8O_5I_2$)], which is a dark green powder and has a molecular weight of 1116.9, was obtained from Porphyrin Laboratory (Okayama, Japan). It was dissolved and diluted in PBS to give a final concentration of 1 or 10 mg/L.

LED system

An LED system (ME-PT-DSRD660-0201) that provides a single light beam with a wavelength of 660 nm was obtained from CCS (Kyoto, Japan). The LED power was measured with an optical power meter (Hioki, Nagano, Japan) during each experiment. The increase in temperature conferred by the LED device was measured with a wire thermometer placed in the culture plate during irradiation. At a distance of 5 cm from the light source, irradiation at a light power of 0.055 W over 3 min is equivalent to a light energy of 10 J/cm^2 at the bottom of the plate. Temperature measurements revealed that continuous LED irradiation increased the temperature of PBS containing the targeted fungal spores and the photosensitizer in wells of the plate to > 40 °C. To avoid this potentially problematic increase in temperature, we included a 1-min rest period between 3-min light exposures.

PACT

An experimental overview and workflow for the study are shown in Fig. 1. We evaluated the effect of TONS 504 on *F. solani* and *A. fumigatus* without LED irradiation, the effect of LED irradiation in the absence of TONS 504, and the effect of the combination of TONS 504 and LED irradiation (TONS 504–PACT). The fungal spores (3000 CFU) and TONS 504 (1 or 10 mg/L) in a total volume of 1 mL were placed in the wells of a 24-well plate. The contents of the wells were exposed to the LED at 10 J/cm^2 (single 3-min exposure), 20 J/cm^2 (two 3-min exposures separated by a 1-min rest period), or 30 J/cm^2 (three 3-min exposures with two 1-min rest periods). After LED irradiation, 100 μL of the well contents (300 CFU of fungal spores) were transferred to a PDA plate (100 mm in diameter) and incubated for 3 to 4 days at 25 °C, after which the number of colonies was counted (this incubation time allowed colony enumeration before hypha formation rendered it too difficult).

Statistical analysis

Quantitative data are presented as means + SD for five plates in each group and were compared among groups with one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. A P value of < 0.01 was considered statistically significant. Statistical analysis was performed with JMP software version 11.0.0 (SAS Institute, Cary, NC, USA).

Results

Antifungal effect of TONS 504–PACT on *F. solani*

Exposure of *F. solani* to TONS 504 alone had no effect on colony formation compared with that apparent for spores not subjected to any treatment (Figs. 2 and 3). LED irradiation alone had only a small inhibitory effect on colony formation, although this effect was statistically significant at 20 and 30 J/cm^2. In contrast, TONS 504–PACT markedly attenuated colony formation by *F. solani*, with this effect being complete with TONS 504 at a concentration of 1 mg/L and LED irradiation at 30 J/cm^2 as well as at a TONS 504 concentration of 10 mg/L and LED irradiation at 10, 20, or 30 J/cm^2.

Antifungal effect of TONS 504–PACT on *A. fumigatus*

Neither TONS 504 nor LED irradiation alone had a significant effect on colony formation by *A. fumigatus* compared with that apparent for spores not subjected to any treatment (Figs. 2 and 3). In contrast, TONS 504–PACT significantly inhibited colony formation at a TONS 504 concentration of 10 mg/L and LED irradiation at 20 or 30 J/cm^2, although this inhibitory effect was not complete at either light energy.

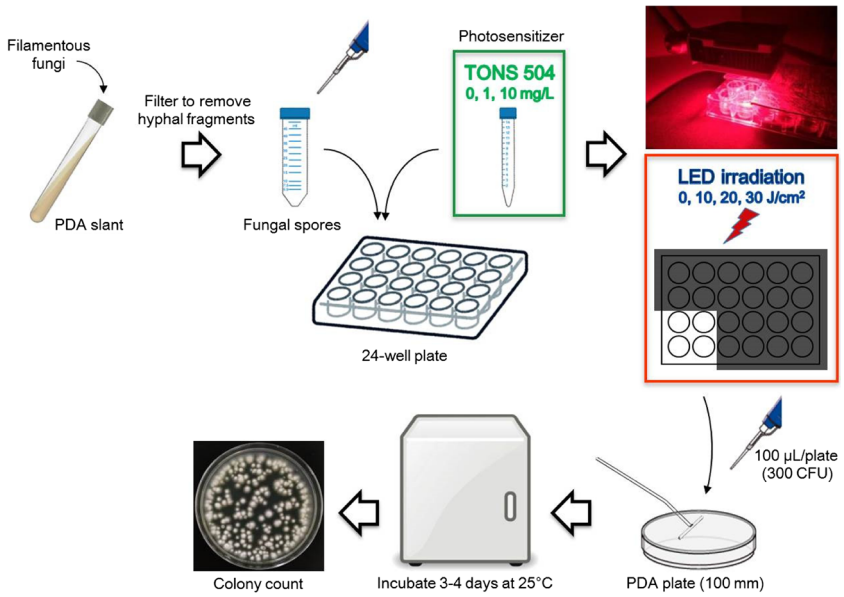

Fig. 1 Experimental overview and workflow

Discussion

We have here shown that TONS 504–PACT was cytotoxic for the filamentous fungi *F. solani* and *A. fumigatus*. We previously showed that this PACT system based on the novel chlorin derivative TONS 504 as the photosensitizer and LED irradiation centered on a wavelength of 660 nm inhibits the proliferation of Gram-positive bacteria including both methicillin-sensitive and methicillin-resistant *S. aureus* as well as that of the Gram-negative bacterium *P. aeruginosa* in vitro with a high potency and efficacy [12, 13]. It can be difficult to evaluate the antimicrobial effect of PACT on filamentous fungi because of the extension of hyphae and clump formation, but we were able to achieve this evaluation with the use of a spore suspension prepared with a surfactant to separate the spores.

The antimicrobial action of PACT is thought to result from injury to the cell wall or cell membrane and DNA damage in the target organism caused by reactive oxygen species, in particular by singlet oxygen [15–17]. Whereas bacteria are prokaryotes and therefore contain naked DNA in their cells, fungi are eukaryotes whose DNA is present within a nucleus and which also possess both a cell membrane and rigid cell wall. Nevertheless, TONS 504–PACT also manifested an antimicrobial action against the two filamentous fungal species examined in the present study. The surface of microorganisms is negatively charged in the physiological environment and most photosensitizers used

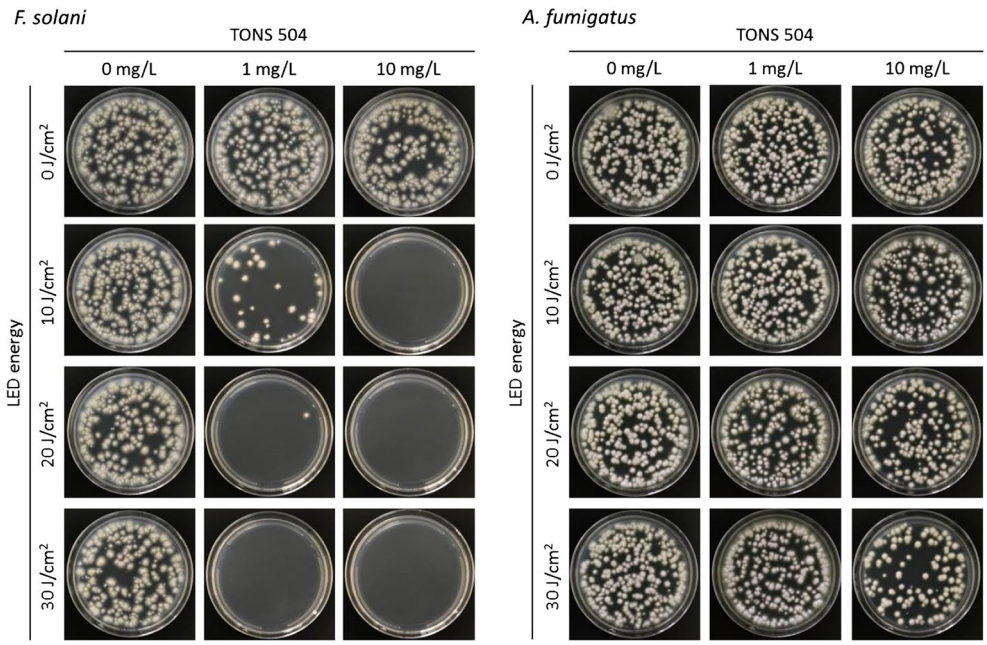

Fig. 2 Representative photographs of colonies formed by *F. solani* and *A. fumigatus* after TONS 504–PACT

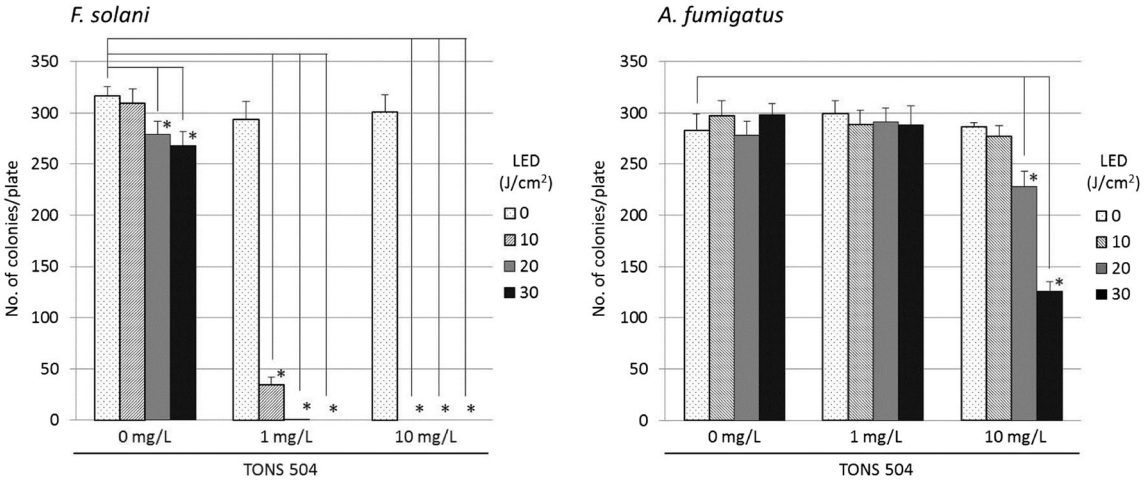

Fig. 3 Quantitation of colony formation by *F. solani* and *A. fumigatus* after TONS 504–PACT. Data are means + SD for five plates corresponding to five independent experiments. $*P < 0.01$ (one-way ANOVA followed by Dunnett's post hoc test)

for PDT are anionic porphyrins or chlorin derivatives, with their anionic nature imposing limitations to their effectiveness [18, 19]. In contrast, TONS 504 is a cationic chlorin derivative, and the emission intensity of singlet oxygen generated by excitement of TONS 504 is twice that for methylene blue. These properties might thus contribute to the effectiveness of TONS 504–PACT against *F. solani* and *A. fumigatus*.

In the case of *F. solani*, complete sterilization was achieved by TON 504–PACT with TONS 504 at a concentration of 1 mg/L and LED irradiation at 30 J/cm^2 as well as with TONS 504 at a concentration of 10 mg/L and LED irradiation at 10, 20, or 30 J/cm^2. LED irradiation alone at 20 or 30 J/cm^2 also showed a significant inhibitory effect on colony formation by *F. solani*, although the mechanism of this effect remains unclear. In the case of *A. fumigatus*, TONS 504–PACT showed a significant effect on viability only at a TONS 504 concentration of 10 mg/L and LED irradiation at 20 or 30 J/cm^2. Furthermore, this cytotoxic effect was not complete. The antifungal effect of PACT on *A. fumigatus* was thus inferior to that on *F. solani*. Organisms express various antioxidant enzymes such as superoxide dismutase and catalase in order to defend against reactive oxygen species that are generated in the process of respiration [20–23]. Whereas most yeast harbor only one catalase gene, many filamentous fungi possess multiple catalase genes [24–27]. Differences in the number of such genes among fungi may contribute to differences in PACT efficacy.

Drug-resistant bacteria that have arisen as a result of the overuse of antibiotics have become a serious clinical problem with regard to the treatment of infectious diseases including infectious keratitis. Such resistance is also a growing problem with regard to the efficacy of antifungal drugs for major pathogenic fungi [28, 29]. We have previously shown the effectiveness of TONS 504–PACT for the elimination of both methicillin-resistant *S. aureus* and acyclovir-resistant herpes simplex virus type 1 [12, 14]. As far as we are aware, there has been no report that repeated application of PACT results in the development of resistance to its antimicrobial effect. PACT is therefore a potential new mode of treatment for infectious keratitis caused by various microorganisms including drug-resistant bacteria, viruses, and fungi.

In conclusion, we have demonstrated the efficacy of PACT with the chlorin derivative TONS 504 and an LED device for elimination of the filamentous fungi *F. solani* and *A. fumigatus*. Further studies are thus warranted to evaluate the effect of TONS 504–PACT on experimental fungal keratitis in vivo.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals.

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