ORIGINAL ARTICLE

Time-dependent antimicrobial effect of photodynamic therapy with TONS 504 on *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa (*P. aeruginosa*) is a major cause of infectious keratitis, which itself is a major cause of blindness worldwide. We have now evaluated the time-dependent effectiveness of photodynamic antimicrobial chemotherapy (PACT) with the chlorin derivative TONS 504 and a light-emitting diode (LED) on *P. aeruginosa* in vitro. PACT with TONS 504 (10 mg/L) and irradiation (30 J/m^2) by an LED device that delivers light centered on a wavelength of 660 nm was applied to 1 × 10^6 colony-forming units of *P. aeruginosa* in liquid medium. The bacteria were then cultured at 37°C for various times before assay of viability by determination of colony formation on agar plates. The effect of a second irradiation at 3 hours after the initial LED exposure was also examined. Bacterial growth was markedly inhibited between 3 and 9 hours after PACT with TONS 504, with the maximal effect being apparent at 3 hours. Furthermore, a second exposure to LED irradiation at 3 hours after the first treatment enhanced the inhibitory effect on bacterial growth. PACT with TONS 504 thus inhibited the growth of *P. aeruginosa* in a time-dependent manner, and an additional irradiation exposure applied 3 hours after the first LED treatment greatly increased the effectiveness of PACT. This antibacterial system thus warrants further evaluation with regard to its potential effectiveness for the treatment of infectious keratitis.

Keywords: Photodynamic antimicrobial chemotherapy (PACT), *Pseudomonas aeruginosa*, Chlorin derivative, TONS 504, Light-emitting diode (LED), Infectious keratitis

Introduction

Infectious keratitis can lead to corneal perforation or scarring and is a major cause of blindness worldwide [1]. *Pseudomonas aeruginosa* (*P. aeruginosa*) is a causative agent for infectious keratitis and the most common cause of this condition related to contact lens wear, with such infection rapidly resulting in impairment of corneal transparency and damage to corneal structure in association with a high level of inflammation. New modes of treatment for infectious keratitis caused by *P. aeruginosa* are urgently needed.

Photodynamic therapy (PDT) has been developed to destroy cancer cells or to induce regression of new blood vessels through generation of reactive oxygen species due to the interaction of irradiating light of a specific wavelength with a photosensitizer accumulated in the target cells [2, 3]. This approach has already been applied to the treatment of various types of cancer 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 subsequent development 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].

We recently showed that PACT with a newly developed chlorin derivative, TONS 504, as the photosensitizer and a light-emitting diode (LED) as the light source was effective for the elimination of both methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* (*S. aureus*) in vitro [12]. The antimicrobial action of PACT is thought to be due to injury to the cell wall or cell membrane of the target organism caused by reactive oxygen species, especially singlet oxygen [13–15]. We have now investigated the time-dependent effectiveness of PACT for elimination of *P. aeruginosa*.

Materials and Methods

Microorganisms

A strain of *P. aeruginosa* (NBRC 13275) obtained from NITE Biological Resource Center was grown in liquid medium overnight at 37°C inside a shaking incubator. The liquid medium consisted of a combination of polypepton (Nihon Pharmaceutical Co. Ltd., Tokyo, Japan), dried yeast extract, and magnesium sulfate heptahydrate (Nacalai Tesque, Kyoto, Japan). The cells were harvested by centrifugation (3000 × *g* for 10 min at 4°C), washed three times with phosphate-buffered saline (PBS), and suspended in PBS at a density of 1×10^8 colony-forming units (CFU)/mL.

Photosensitizer

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-methy iodide ($C_{51}H_{58}N_8O_5I_2$)], a hydrophilic and cationic chlorin derivative with a greenish color and molecular weight of 1116.9 (Fig. 1), was obtained from Porphyrin Laboratory (Okayama, Japan). It was dissolved in a small amount of sterile double-distilled water and diluted with liquid medium to a concentration of 10 mg/L.

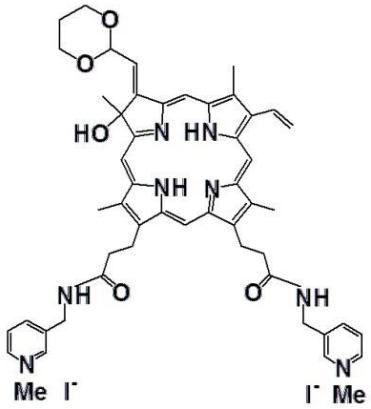

Fig. 1 Chemical structure of the cationic chlorin derivative TONS 504

LED system

An LED system (ME-PT-DSRD660-0201) that delivers light centered on a wavelength of 660 nm (Fig. 2) 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. We found that irradiation at a distance of 5 cm from the light source to the bottom of the plate yielded a light power of 0.055 W (spot diameter of 23 mm) and light energy of 10 J/cm^2 over 3 min. On the basis of the temperature measurements, we included a 1-min rest period between 3-min light exposures in order to avoid a potentially problematic increase in temperature of the liquid medium.

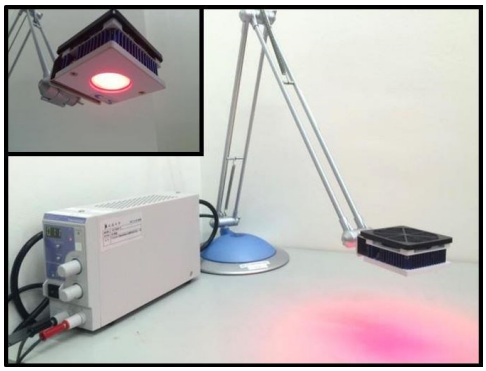

Fig. 2 The LED system, with the light-emitting component shown in the inset

PACT

The experimental overview and workflow for the study are shown in Figure 3. We evaluated the effect of TONS 504 on *P. aeruginosa* without LED exposure, the effect of LED exposure in the absence of TONS 504, and the effect of the combination of TONS 504 and LED exposure (TONS 504–PACT). The five experimental groups were thus as follows: (1) no treatment (n = 3), (2) TONS 504 alone (n = 3), (3) LED irradiation alone (n = 3), (4) TONS 504–PACT (n = 4), and (5) TONS 504–PACT with an additional irradiation exposure after 3 h (n = 3).

Bacteria (1×10^8 CFU/mL) in 10 μL of PBS were transferred to the wells of a 24-well plate, 1 mL of TONS 504 (10 mg/L) in liquid medium was added to each well, and the plate was incubated for 5 min at room temperature before exposure to the LED at 30 J/cm^2 (three 3-min exposures separated by two 1-min rest periods). The plate was then incubated at 37°C. At 0, 0.5, 1, 3, 6, 9, 12, and 24 hours after LED irradiation, 100 μL of the contents of a well (1×10^5 CFU of bacteria) were transferred to an agar plate (100 mm in diameter, containing agar [Nacalai Tesque] in liquid medium). The agar plates were incubated for 36 hours at 37°C, after which visible colonies were counted. In the case of experimental group 5, a second LED irradiation (30 J/cm^2) was performed (without further addition of TONS 504) at 3 hours after the initial exposure, and colony formation was evaluated after the same time intervals (0 to 24 hours) as described above.

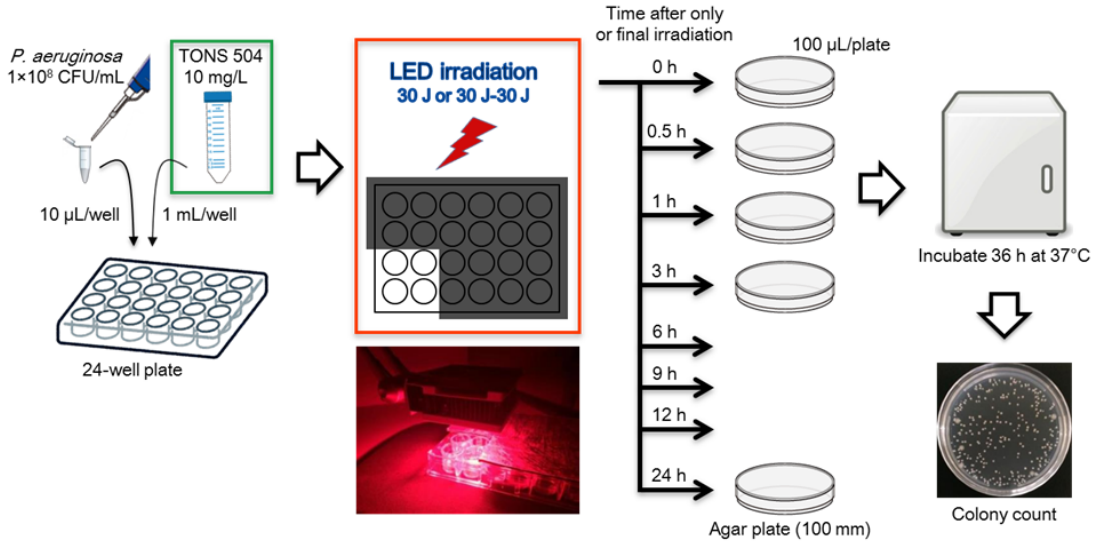

Fig. 3 Experimental overview and workflow

The bacteria and photosensitizer are transferred to the wells of a 24-well plate, and the mixture in each well is then exposed to LED irradiation (or not, as a control). The plate is incubated for various times before transfer of a portion of each mixture to an agar plate, which is then incubated for 36 hours at 37°C for colony enumeration.

Results

The results of the colony formation assay with agar plates are shown visually in Figure 4, with the number of colonies being presented in Table 1. Bacteria treated with either TONS 504 (group 2) or LED irradiation (group 3) alone manifested no inhibition of their growth compared with that apparent for bacteria not subjected to any treatment (group 1). In contrast, bacteria subjected to TONS 504–PACT (group 4) showed a reduction in viability that was not apparent until 1 hour after irradiation and was most pronounced at 3 to 9 hours. The maximum inhibitory effect was actually observed at 3 hours after irradiation, when the bacterial count had declined from 1×10^5 CFU to 93.2 ± 16.7 CFU. In the case of group 5, in which bacteria were subjected to TONS 504–PACT but also underwent a second exposure to LED irradiation 3 hours later, the inhibition of bacterial growth was even greater, being almost complete between immediately and 3 hours after the second irradiation. Even at 6 h after the additional irradiation, the number of bacteria was still markedly low at 170.7 ± 29.5 CFU.

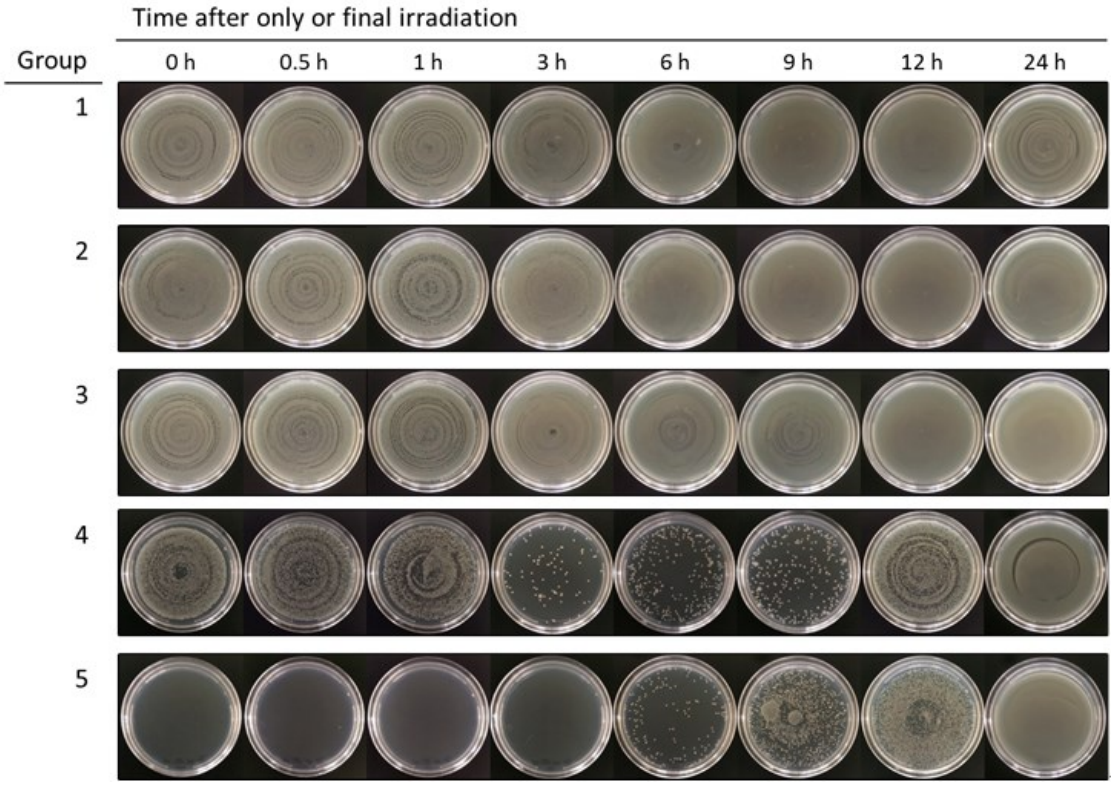

Fig. 4 Photographs of colonies formed by bacteria in the five experimental groups. Groups: (1) no treatment, (2) TONS 504 exposure alone, (3) LED irradiation alone, (4) TONS 504 exposure plus LED irradiation (TONS 504–PACT), and (5) TONS 504–PACT plus a second irradiation 3 hours after the first.

Table 1 Number of colonies formed by bacteria in the five experimental groups

Experimental group	Time after irradiation (second irradiation in group 5)							
	0 h	0.5 h	1 h	3 h	6 h	9 h	12 h	24 h
1	+++	+++	+++	+++	+++	+++	+++	+++
2	+++	+++	+++	+++	+++	+++	+++	+++
3	+++	+++	+++	+++	+++	+++	+++	+++
4	+++	+++	+++	93.2 ± 16.7	294.0 ± 15.8	403.3 ± 46.2	+++	+++
5	0.33 ± 0.6	1.33 ± 0.6	1.0 ± 1.7	1.67 ± 1.5	170.7 ± 29.5	+++	+++	+++

Data are means ± SD from four independent experiments. Groups: (1) no treatment, (2) TONS 504 exposure alone, (3) LED irradiation alone, (4) TONS 504 exposure plus LED irradiation (TONS 504–PACT), and (5) TONS 504–PACT plus a second irradiation 3 hours after the first. +++ indicates >500 CFU.

Discussion

We have here shown that TONS 504–PACT was effective against the Gram-negative bacterium *P. aeruginosa*. We previously showed that this PACT system based on the novel chlorin derivative TONS 504 as the photosensitizer and LED-based irradiation centered on a wavelength of 660 nm inhibits the proliferation of Gram-positive bacteria including both methicillin-sensitive and methicillin-resistant *S. aureus* in vitro with a high potency and efficacy [12]. PACT is thought to be less effective against Gram-negative bacteria than Gram-positive bacteria as a result of the difference in cell membrane and cell wall structure [16]. Gram-positive bacteria have a cytoplasmic membrane and thick peptidoglycan layer, whereas Gram-negative bacteria possess inner and outer membranes separated by a thin peptidoglycan layer. The porous nature of the cell wall structure of Gram-positive bacteria allows penetration by photosensitizers. On the other hand, the abundance of lipopolysaccharide in the outer membrane of Gram-negative bacteria impedes photosensitizer penetration [17, 18]. In addition, the surface of microorganisms is negatively charged in the physiological environment and most photosensitizers used for PDT are anionic porphyrins or chlorin derivatives, with such compounds being less effective against Gram-negative bacteria [19, 20]. In contrast, TONS 504 is a cationic chlorin derivative, and the emission intensity of singlet oxygen generated by excitement of TONS 504 is twice as high as that for methylene blue. We therefore hypothesized that TONS 504–PACT might also be effective against Gram-negative bacteria such as *P. aeruginosa*.

We found that the antibacterial effect of TONS 504–PACT in the present study was not instantaneous but developed in a time-dependent manner. The antimicrobial action of PACT is thought to be mediated predominantly by the generation of singlet oxygen and other reactive oxygen species and consequent injury to the cell wall or cell membrane of the target organism [13-15]. Evaluation of PACT is often based on monitoring of the growth of microorganisms that have been seeded on an agar plate and then irradiated [21–23]. However, this approach does not allow characterization of the time course of the antimicrobial effect. In the present study, we were able to evaluate the time-dependent action of TONS 504–PACT by irradiating the bacteria in liquid medium and then monitoring colony formation at various times thereafter by transfer of culture samples to agar plates. Although reactive oxygen species are generated instantaneously on irradiation of a photosensitizer with light of the appropriate wavelength, we found that the antimicrobial effect of TONS 504–PACT against *P. aeruginosa* was not apparent until 1 hour after irradiation. PDT has been shown to induce apoptosis in tumor cells [24, 25]. Prokaryotes have also been found to undergo death by a mechanism similar to apoptosis known as the toxin-antitoxin system, with such

programmed cell death being thought to be induced by various types of stress [26, 27]. With regard to the time-dependent effect of TONS 504–PACT, it is thus possible that the stress associated with exposure to reactive oxygen species also induces death of bacteria via the toxin-antitoxin system.

We found that an additional LED irradiation at 3 hours after TONS 504–PACT enhanced the inhibitory effect on bacterial growth. This enhanced effect is likely due to bacteria that survive the initial PACT being injured by singlet oxygen generated by excitation of remaining photosensitizer. Bacteria that survive TONS 504–PACT with or without the second irradiation are able to proliferate because they are maintained in nutrient-rich liquid growth medium. Although additional TONS 504–PACT treatments might be expected to completely eliminate the bacteria, such a complete bactericidal effect might not be necessary in the in vivo setting because of the biological reactions that also target infection.

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. We have previously shown the effectiveness of PACT against methicillin-resistant *S. aureus* as well as against acyclovir-resistant herpes simplex virus type 1 [12, 28]. As far as we are aware, there has been no report that repeated application of PACT results in the development of resistance to its antibacterial effect. PACT is therefore a potential new mode of treatment for infectious keratitis caused by various microorganisms including drug-resistant bacteria.

In conclusion, we have demonstrated a time-dependent antimicrobial effect of PACT with the chlorin derivative TONS 504 and a LED system against *P. aeruginosa*. Further studies are thus warranted to evaluate the effect of TONS 504–PACT on experimental bacterial 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

All experimental protocols were approved by the Hiroshima University Animal Experiment Committee, and were in keeping with the basic guidelines of Hiroshima University regarding animal experiments (Approval number: A15-76).

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