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Antimicrobial Photodynamic Therapy with the photosensitizer TONS504 eradicates *Acanthamoeba*

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ABSTRACT

Background: Microbial keratitis is a potential cause of corneal blindness. We investigated the amoebicidal efficacy of photodynamic antimicrobial therapy with a light-emitting diode as the light source and the cationic chlorin derivative TONS504 as the photosensitizer for the elimination of *Acanthamoeba*, a causative organism of corneal infection and blindness. Acanthamoeba keratitis remains a challenge to treat because of limited available treatments.

Methods: *Acanthamoeba castellani* 50370 was exposed to TONS504 at various concentrations (0, 1, or 10 mg/L for trophozoites; 0, 1, 10, or 20 mg/L for cysts), irradiated at various light energies (0, 10, or 30 J/cm^2 for trophozoites; 0, 30, or 60 J/cm^2 for cysts), and incubated at 26 °C for 3 h. Assessment of cell viability by trypan blue staining revealed that photodynamic antimicrobial therapy attenuated the survival of trophozoites and cysts dependent on TONS504 concentration and light energy.

Results: Photodynamic antimicrobial therapy with 10 mg/L TONS504 and 30 J/cm^2 light energy suppressed trophozoite viability by 77%, and 20 mg/L TONS504 and 60 J/cm^2 light energy attenuated cyst survival by 42%. Staining with fluorescein isothiocyanate–conjugated annexin V and ethidium homodimer III revealed photodynamic antimicrobial therapy induced apoptosis *and* necrosis in trophozoites dependent upon the intensity of treatment, whereas apoptosis was the predominant form of cell death in cysts.

Conclusions: Photodynamic antimicrobial therapy with TONS504 warrants further investigation as a potential treatment modality for *Acanthamoeba* keratitis.

1. Introduction

Acanthamoeba keratitis is a painful and sight-threatening infection of the cornea caused by species of *Acanthamoeba*, a free-living amoeba that exists in an active trophozoite stage and a dormant cyst stage. It is highly associated with ocular trauma and exposure to contaminated water, often occurring as a result of poor hygiene in wearers of contact lenses [1]. The incidence of Acanthamoeba keratitis on the previous reports is widely variable around the countries. In the United States, the Acanthamoeba keratitis incidence was 0.15–2.01 per million and 1.4–42 cases per million inhabitants in the United Kingdom [2,3]. Treatment of *Acanthamoeba* keratitis is challenging because of the limited number of available drugs that are active against this microorganism. Until now, there are no drugs specifically approved by the FDA for treating Acanthamoeba keratitis. The recommended treatment is debridement of focal lesions combined with the administration of antifungal drugs including primary biguanides (polyhexamethylene biguanide and chlorhexidine) and diamidines (propamidine isethionate and hexamidine). However, harmful effects on the cornea and resistance to these agents may constrain the effectiveness of this treatment [4,5].

Photodynamic therapy was recently developed as a therapeutic modality with minimal invasiveness and low toxicity for oncological disease. Photodynamic therapy consists of the administration of a

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photosensitizer that is taken up by the malignant tissue followed by irradiation with light of an appropriate wavelength that results in the death of the targeted tumor cells or damage to the associated vasculature [6,7]. Photodynamic therapy has also historically been applied to the inactivation of microorganisms, for which it is termed Antimicrobial Photodynamic Therapy. The recent increase in microbial resistance to antibiotics and other drugs has led to a resurgence of interest in Antimicrobial Photodynamic Therapy as an alternative antimicrobial therapy. Upon irradiation of the photosensitizer at a specific wavelength corresponding to its absorption peak, the excited molecule reacts with molecular oxygen or other objects in biological systems by electron transfer to generate radical species, known as Type I mechanism. Alternatively, in the type II mechanism, the excitation energy can be transferred from the excited triplet of the photosensitizer to triplet dioxygen, which returns the photosensitizer to the ground state and generates excited singlet oxygen. The accumulation of radical and singlet oxygen species causes irreversible damage to the targeted microbial cell [8–11].

We have been developing Antimicrobial Photodynamic Therapy as a potential new treatment for infectious keratitis. We previously showed that Antimicrobial Photodynamic Therapy with a light-emitting diode as the light source and the novel chlorin derivative TONS504 as the photosensitizer was effective for the elimination of methicillin-sensitive and methicillin-resistant strains of *Staphylococcus aureus, Pseudomonas aeruginosa*, herpes simplex virus type 1, and pathogenic fungi *in vitro* [12–15]. Here, we examined whether the Antimicrobial Photodynamic Therapy system (TONS504–PACT) might also be effective against *Acanthamoeba*.

2. Materials and methods

2.1. Acanthamoeba isolate and culture

Acanthamoeba castellani (American Type Culture Collection 50370) was grown in a xenic culture at 26 °C with a lawn of heat-inactivated *Escherichia coli* (American Type Culture Collection 8739) in 10-cm tissue culture petri dishes (Corning, Corning, NY) containing CHROMagar Candida Medium (Kanto Chemical, Tokyo, Japan). After culture for 5 days, trophozoites were washed with *Acanthamoeba* saline [0.012% *Natrium Chloride*, 0.00035% *Potassium Chloride*, 0.0003% *Calcium chloride*, and 0.0004% *Magnesium chloride* heptahydrate in 0.05 mM *Tris-hydrochloride* (pH 6.8)], isolated by centrifugation at 1000 × *g* for 10 min, suspended in the saline solution, and adjusted to a final concentration 1×10^6/mL. Encystment was induced by the transfer of trophozoites from CHROMagar to *Acanthamoeba* saline and incubation for at least 2 weeks at 26 °C.

2.2. Photosensitizer

TONS504 [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 (C51H58O5I2)] was provided by the Porphyrin Laboratory (Okayama, Japan). This hydrophilic and cationic chlorin derivate has a molecular weight of 1116.9 and is greenish in color (Fig. 1A). This photosensitizer has an absorption spectrum about 667 nm (Fig. 1B). It was dissolved in *Acanthamoeba* saline and serially diluted to the desired concentrations.

2.3. LED system

The light source was a light-emitting diode system (ME-PT-DSRD660-0201) provided by CCS (Kyoto, Japan) that emits light centered at a wavelength of 660 nm (Fig. 2). The light-emitting diode power was measured with an optical power meter (Hioki, Nagano, Japan). The increased temperature was confirmed by measured with a wire thermometer placed in the culture plate during irradiation. The

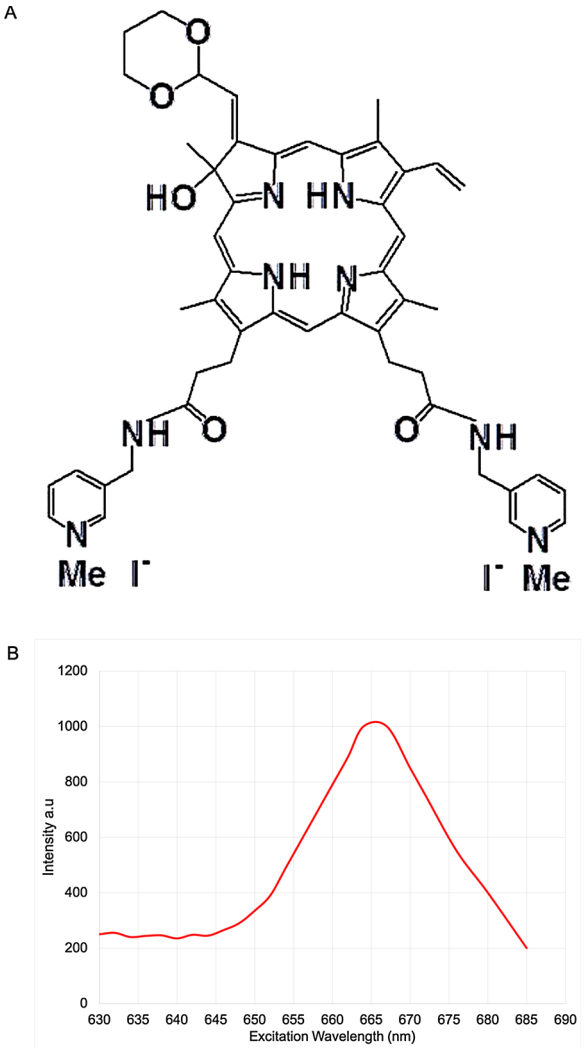

Fig. 1. Chemical structure of cationic chlorin derivative TONS504 (A) and its absorption spectrum (B).

results revealed that irradiation at a distance 5 cm from the source of light to the bottom of the plate yielded a light energy of 10 J/cm^2 and light power of 0.055 W (spot diameter of 23 mm) over 3 min. In order to avoid a problematic due to increase in temperature, we added a 1-min rest period between each 3-min light exposure.

2.4. TONS504–PACT

Portions (10 μL) of *Acanthamoeba* trophozoites or cysts (1×10^6/mL) in *Acanthamoeba* saline were transferred to 35-mm Petri dishes (Corning). TONS504 (1000 μL) at various concentrations (0, 1, or 10 mg/L for trophozoites, or 0, 1, 10, or 20 mg/L for cysts) in *Acanthamoeba* saline was then added to each dish, and the dishes were placed uncovered below the light-emitting diode system for irradiation at 10 J/cm^2 (single 3-min exposure), 30 J/cm^2 (three 3-min exposures separated by two 1-min rest periods), or 60 J/cm^2 (six 3-min exposures separated by five 1-min rest periods). The control (TONS504 0 mg/L, light-emitting diode 0 J/cm^2) and treated groups were then cultured at 26 °C for 3 h before measurement of viability by staining with trypan blue or detection of apoptosis or necrosis by fluorescence microscopic analysis.

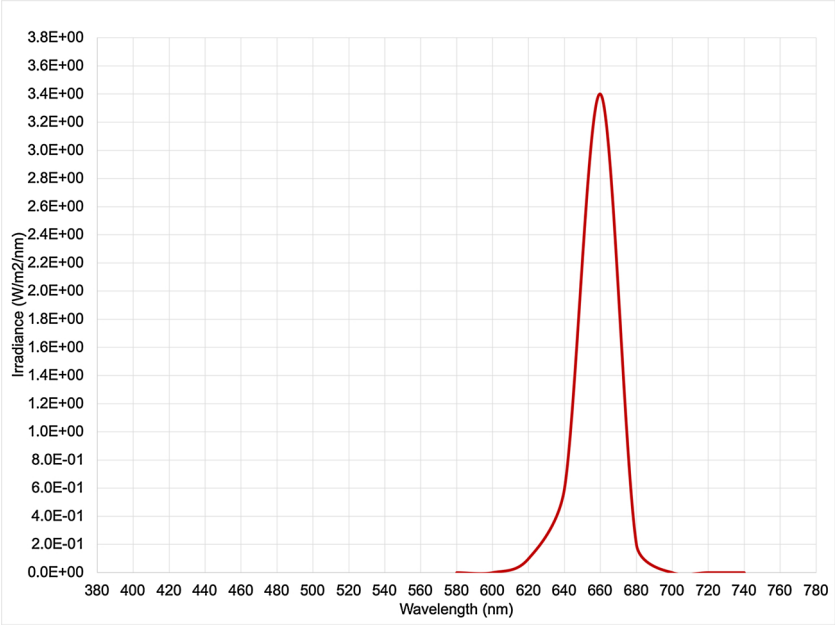

Fig. 2. LED emission spectrum. Emission spectrum of LED used (in arbitrary units (AU) of power (W)). The dominant wavelength is 660 nm LED emission spectrum.

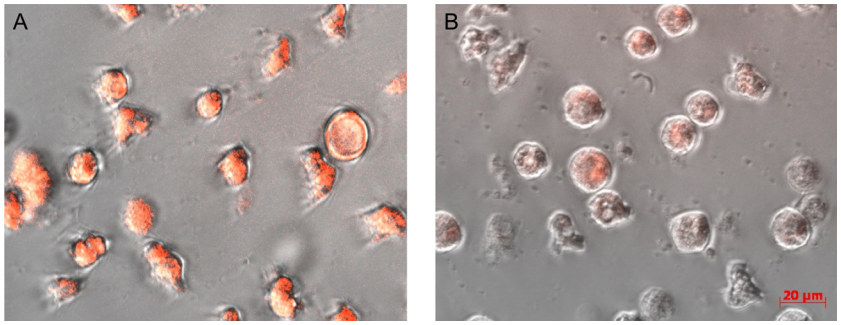

Fig. 3. Localization of TONS504 in *Acanthamoeba*. *Acanthamoeba* trophozoites (A) or cysts (B) were exposed to TONS504 (10 mg/L) in *Acanthamoeba* saline for 1 h at 37 °C and were then examined for the red fluorescence of TONS504 (with Alexa filter 647 nm). The photosensitizer was detected within the organelles of trophozoites, and around the surface and within the double wall of cysts. Scale bar: 20 µm.

2.5. Trypan blue staining

The viability of *Acanthamoeba* trophozoites or cysts was determined on the basis of trypan blue exclusion. Portions (10 µL) of the treated cells were transferred aseptically to 1.5-mL Eppendorf tubes and incubated for 1 min at room temperature with an equal volume of 0.4% trypan blue solution. The cells were then counted with the use of a hemocytometer and observed with a light microscope. Viable and nonviable cells were counted separately and the viable cell ratio was calculated [16].

2.6. Fluorescence microscopic analysis of cell death

After TONS504–PACT, *Acanthamoeba* trophozoites or cysts in the 35-mm petri dishes were washed twice with phosphate-buffered saline, isolated by centrifugation at 1000 × *g* for 10 min, and transferred to 15-mm coverslips coated with poly-L-lysine and placed in the wells of a 24-well culture plate. Apoptotic and necrotic cells were then detected with the use of a Promokine Apoptotic/Necrotic Cells Detection Kit (Promocell, Heidelberg, Germany), which stains apoptotic cells green with fluorescein isothiocyanate–conjugated annexin V and late apoptotic and necrotic cells red with ethidium homodimer III. The stained cells were examined with a laser-scanning fluorescence microscope (Axio Observer D1 Inverted Microscope; Carl Zeiss, Jena, Germany) equipped with AxioVision software (version 4.8, Carl Zeiss).

2.7. Statistical analysis

Data are presented as the means ± standard deviation for trophozoites or as the median (range) for cysts. Differences among treatment groups were evaluated by one-way analysis of variance followed by Dunnett's test for trophozoites, or by the Kruskal-Wallis test followed by the Mann-Whitney *U*-test for cysts. A *P*-value of < 0.05 was considered statistically significant.

3. Results

3.1. Localization of TONS504 in Acanthamoeba

Acanthamoeba trophozoites or cysts were incubated with TONS504 (10 mg/L) for 1 h and were then examined by fluorescence microscopy to evaluate the uptake and distribution of the photosensitizer. The red fluorescence of TONS504 was detected within cellular organelles of trophozoites (Fig. 3A) and around the surface and within the double wall of cysts (Fig. 3B).

3.2. TONS504–PACT decreases the viability of Acanthamoeba trophozoites

Whereas trypan blue staining revealed that the survival rate of *Acanthamoeba* trophozoites was not affected by light irradiation (10 or 30 J/m^2) or TONS504 (1 or 10 mg/L) alone, TONS504–PACT attenuated the viability of trophozoites dependent on TONS504 concentration and light energy (Fig. 4). At a TONS504 concentration of

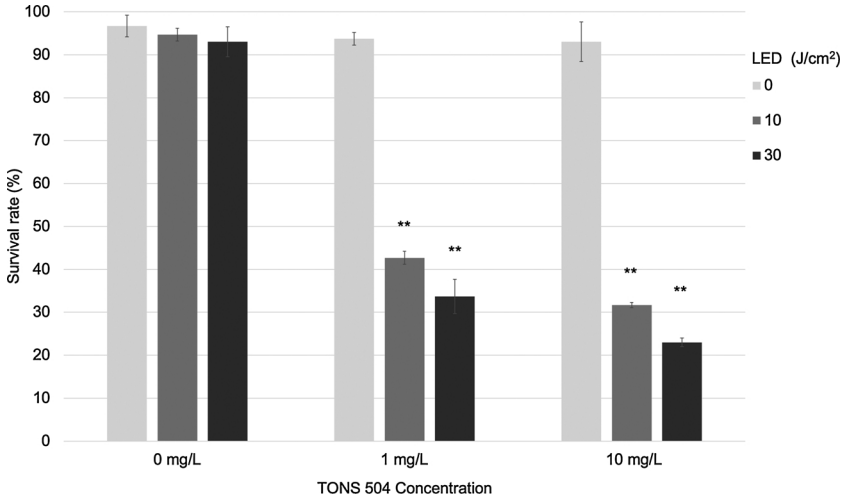

Fig. 4. Effect of TONS504–PACT on the viability of *Acanthamoeba* trophozoites. *Acanthamoeba* trophozoites were subjected to Antimicrobial Photodynamic Therapy at the indicated light irradiation energies and TONS504 concentrations, and their survival rate was determined 3 h later by trypan blue staining. Data are the means ± standard deviation for three independent experiments, each performed in triplicate. $^{**}P < 0.01$ *versus* the value for untreated cells (one-way analysis of variance followed by Dunnett's test).

1 mg/L, irradiaztion at 10 or 30 J/cm^2 resulted in a significant decrease in the survival rate to 42.7 ± 1.5% ($P < 0.01$) and 33.7 ± 4.0% ($P < 0.01$), respectively. At a TONS504 concentration of 10 mg/L, light exposure at 10 or 30 J/cm^2 resulted in a significant decrease in the survival rate to 31.7 ± 0.6% ($P < 0.01$) and 23.0 ± 1.0% ($P < 0.01$), respectively.

3.3. TONS504–PACT decreases the viability of Acanthamoeba cysts

Cysts are a dormant stage of *Acanthamoeba* that are more difficult to eradicate than trophozoites. Again, the survival rate of *Acanthamoeba* cysts was not affected by light irradiation (30 or 60 J/m^2) or TONS504 (1, 10, or 20 mg/L) alone (Fig. 5). At an irradiation energy of 30 J/cm^2, the survival rates were 86.0% (86.0–89.0%), 76.0% (75.0–80.0%), and 74.0% (71.0–76.0%) at TONS504 concentrations of 1, 10, or 20 mg/L ($P < 0.05$), respectively, whereas at 60 J/cm^2 they were 75.0% (74.0–76.0%) and 58.0% (57.0–59.0%) at TONS504 concentrations of 10 and 20 mg/L ($P < 0.05$), respectively.

3.4. TONS504–PACT on apoptosis and necrosis in Acanthamoeba

Fluorescence microscopic analysis of stained cells was performed to determine whether the cell death induced by TONS504–PACT was mediated by apoptosis or necrosis (Fig. 6). At a TONS504 concentration of 1 mg/L and irradiation energy of 10 J/cm^2, the proportions of apoptotic or necrotic trophozoites were 43.7 ± 6.0% and 15.3 ± 4.7% ($P < 0.05$), respectively (Fig. 7). In contrast, at a TONS504 concentration of 10 mg/L and irradiation energy of 30 J/cm^2, the proportion of apoptotic cells had decreased to 16.3 ± 4.0% ($P < 0.05$) whereas that of necrotic cells had increased to 61.7 ± 5.5% ($P < 0.05$). For cysts, the proportions of apoptotic and necrotic cells were 22.0% (18.0–23.0%) and 1.0% (1.0–2.0%), respectively, at a TONS504 concentration of 10 mg/L and irradiation energy of 30 J/cm^2. At a TONS504 concentration of 20 mg/L and irradiation energy of 60 J/cm^2 the proportions of apoptotic and necrotic cells were 39.0% (39.0–46.0%) and 5.0% (5.0–6.0%) ($P < 0.05$), respectively (Fig. 8).

4. Discussion

This study reports that TONS504–PACT was effective for the eradication of *Acanthamoeba* trophozoites and cysts. These effects were dependent on both the TONS504 concentration and irradiation energy. We previously showed that TONS504–PACT was effective against *S. aureus*, *P. aeruginosa*, herpes simplex virus type 1, and pathogenic fungi [12–15]. In our previous study, TONS504-PACT did not exhibit cytotoxicity in human FL cells (ATCC CCL-62™; American Type Culture Collection, Manassas, VA, USA) at various doses of TONS504 (0.01–10 mg/L) and irradiation energies (10–30 J/cm^2) [14]. The cytotoxicity results were similar for human corneal fibroblast cells (obtained from donor cornea at Hiroshima University Hospital), in which TONS504-PACT did not cause any appreciable cellular damage (data not shown). Our present and previous results support the further development of TONS504–PACT as a potential treatment for infectious keratitis caused by a wide spectrum of microorganisms.

The amoebicidal effect of PACT is based on irradiation of the photosensitizer at an appropriate wavelength [17–19]. Such activation of the photosensitizer generates singlet oxygen and other reactive oxygen species that induce damage to the cell wall or cell membrane, eventually leading to the death of the target pathogen [20,21].

We found that TONS504–PACT eradicated up to 77% of

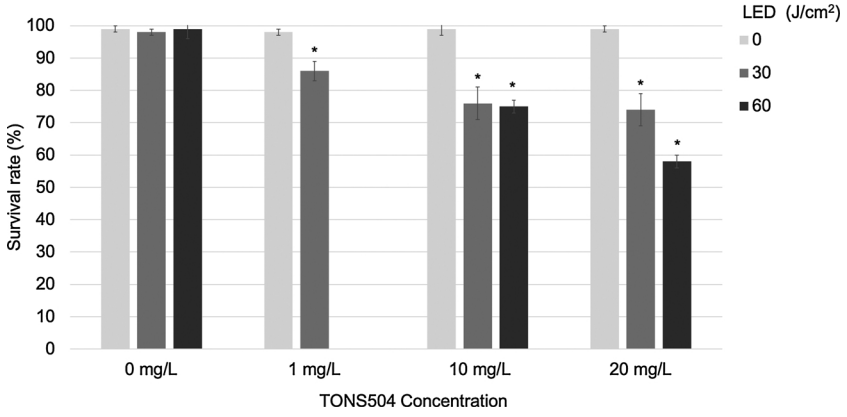

Fig. 5. Effect of TONS504–PACT on the viability of *Acanthamoeba* cysts. *Acanthamoeba* cysts were subjected to Antimicrobial Photodynamic Therapy at the indicated light irradiation energies and TONS504 concentrations, and their survival rate was determined 3 h later by trypan blue staining. Data are presented as the median (range) for three independent experiments, each performed in triplicate. $^{*}P < 0.05$ *versus* the value for untreated cells (Kruskal-Wallis test followed by the Mann-Whitney *U*-test).

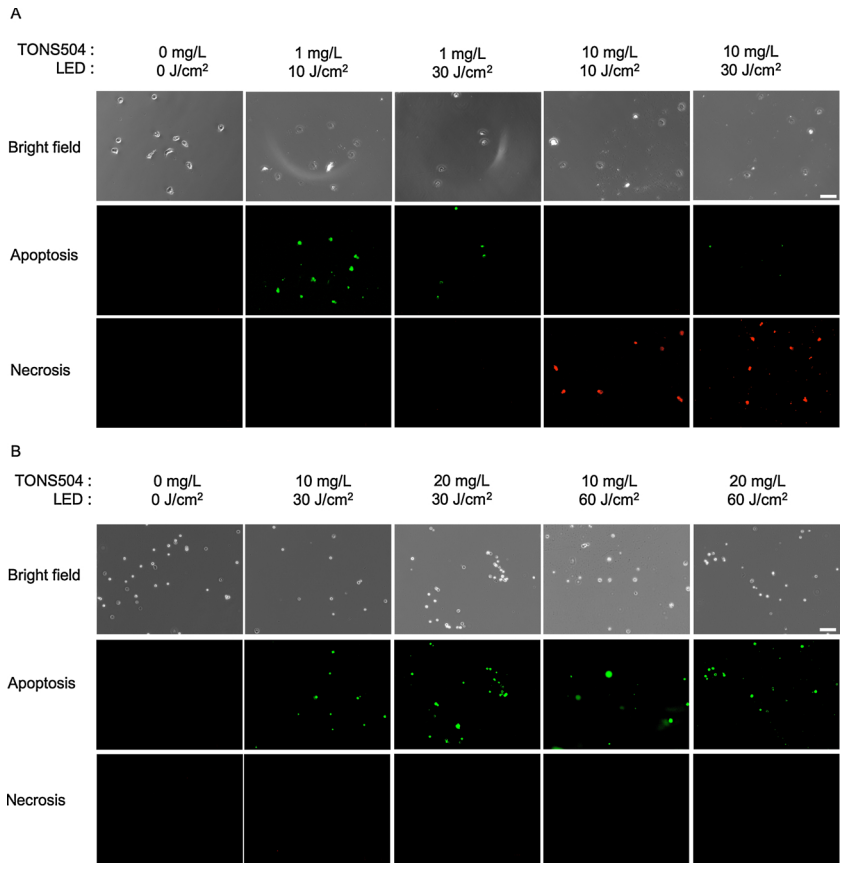

Fig. 6. Fluorescence microscopic analysis of apoptosis and necrosis in *Acanthamoeba* subjected to TONS504–PACT. *Acanthamoeba* trophozoites (A) or cysts (B) were subjected to Antimicrobial Photodynamic Therapy at the indicated light irradiation energies and TONS504 concentrations, and the proportions of apoptotic (green fluorescence) and necrotic (red fluorescence) cells were determined 3 h later with the use of a staining kit and fluorescence microscopy. Scale bars: 50 μm (A) or 100 μm (B).

Acanthamoeba trophozoites. The efficacy of this approach may be due in part to the positive charge of the photosensitizer, which facilitates electrostatic interaction with negatively charged cellular components including the cell wall, mitochondria, nucleus, and lysosomes [22,23]. The emission intensity of singlet oxygen generated by the excitement of TONS504 was twice that observed with methylene blue [13]. TONS504–PACT also attenuated the viability of up to 42% of *Acanthamoeba* cysts. The extent of this effect is surprising given that this dormant stage of the organism is resistant to harsh conditions as a result of its double cell wall, and it may reflect the accumulation of photosensitizer within the cyst wall and its subsequent penetration across the operculum membrane and targeting of the trophozoites located inside the shell [24,25]. TONS504 was shown to localize to lysosomes in mouse mammary tumor cells [26]. This localization may be important for the initiation of cell death [27]. Determination of the precise localization of TONS504 in *Acanthamoeba* trophozoites and cysts may provide further insight into its mechanism of action.

PACT with tetracationic phthalocyanine (RLP068) or perylenequinonoid (hypocrellin B) photosensitizers was previously shown to eradicate *Acanthamoeba*, but the mechanism of microbial inactivation is unclear [25,27]. We investigated the mechanism of cell death induced by TONS504–PACT in *Acanthamoeba* trophozoites and cysts. Apoptosis was detected with the use of fluorescein isothiocyanate–labeled annexin V, which binds to phosphatidylserine translocated from the inner to the outer surface of the plasma membrane in cells undergoing programmed cell death [28]. Necrosis was detected with the use of ethidium homodimer III, which stains *deoxyribonucleic acid* in disrupted cells. TONS504–PACT induced apoptosis *and* necrosis in trophozoites dependent upon the intensity of treatment, whereas apoptosis was the predominant form of cell death in cysts.

In conclusion, TONS504–PACT induced the death of *Acanthamoeba* trophozoites and cysts by a mechanism dependent on TONS504 concentration and light energy. Trophozoites were substantially more susceptible to TONS504–PACT than cysts, likely reflecting the ability of the photosensitizer to bind to or accumulate within the targeted stage of the *Acanthamoeba* life cycle. Our results described an *in vitro* study,

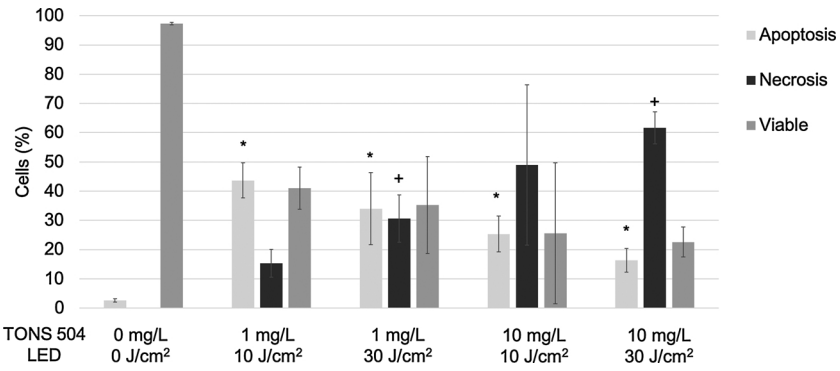

Fig. 7. Effects of TONS504–PACT on apoptosis and necrosis in *Acanthamoeba* trophozoites. *Acanthamoeba* trophozoites were subjected to Antimicrobial Photodynamic Therapy at the indicated light irradiation energies and TONS504 concentrations, and the proportions of apoptotic, necrotic, and viable cells were determined 3 h later. Data are the means ± standard deviation for three independent experiments, each performed in triplicate. $^{*}P < 0.05$ compared with untreated cells; $^{+}P < 0.05$ compared with untreated cells (one-way analysis of variance followed by Dunnett's test).

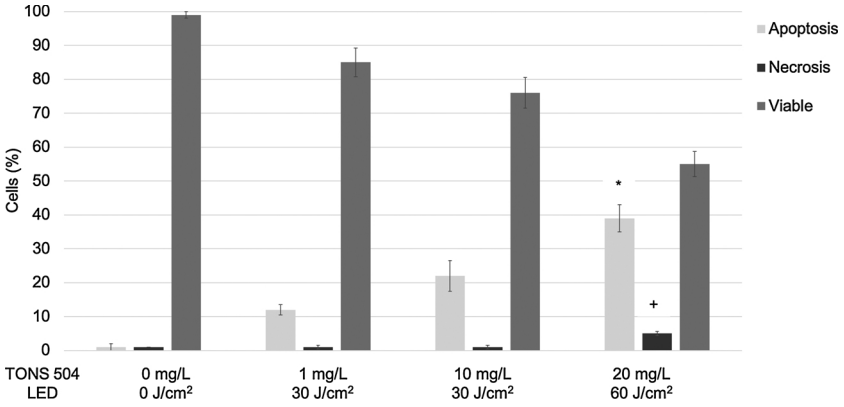

Fig. 8. Effects of TONS504–PACT on apoptosis and necrosis in *Acanthamoeba* cysts. *Acanthamoeba* cysts were subjected to Antimicrobial Photodynamic Therapy at the indicated light irradiation energies and TONS504 concentrations, and the proportions of apoptotic, necrotic, and viable cells were determined 3 h later. Data are presented as the median (range) for three independent experiments, each performed in triplicate. $^{*}P < 0.05$ compared with untreated cells; $^{+}P < 0.05$ compared with untreated cells (Kruskal-Wallis test followed by the Mann-Whitney *U*-test).

which might be different to *ex vivo* or *in vivo* studies. Further studies are thus warranted to evaluate the efficacy of TONS504–PACT for the treatment of experimental *Acanthamoeba* keratitis *in vivo*.

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Declaration of Competing Interest

None.

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