Effect of Photodynamic Therapy with Methylene Blue on Acanthamoeba In Vitro

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PURPOSE. To evaluate the disinfectant effect of methylene blue (MB)-mediated photodynamic therapy (PDT) on a pathogenic strain of Acanthamoeba.

METHODS. Acanthamoeba castellanii (ATCC 50370) used in this study were treated under one of four experimental conditions: light irradiation and incubation in MB (L+M⁺), light irradiation and incubation in physiologic solution (L+M⁻), incubation in MB only (L⁻M⁺), and incubation in physiologic solution (L⁻M⁻). M⁺ trophozoites were incubated in either 0.25 or 0.5 mM MB for 10 minutes. L⁻ organisms were irradiated for 30 minutes following incubation in solution. A halogen lamp (660 ± 10 nm) with a maximum output of 6 mW/cm² was used as the PDT light source. After treatment, antiacanthamoeba activity was evaluated by checking the respiratory activity of the amoeba with 5-cyano-2,3-tetrazolium chloride (CTC) staining. We also determined whether the effect of PDT with MB had been retained or augmented when it was performed in combination with conventional antiamoebic agents.

RESULTS. MB-PDT suppressed the respiratory activity of trophozoites in an MB-concentration-dependent manner at total light doses of 10.8 J/cm². The respiratory activity of each group as a percentage of that of L⁻M⁻ is as follows: L⁻M⁺: 11.6% (0.5 mM), 60.9% (0.25 mM); L⁻M⁻: 116.5% (0.5 mM), 105.5% (0.25 mM); L⁺M⁻: 107.6%; and L⁺M⁻: 106.3% (L⁺M⁻ versus L⁻M⁻ P < 0.05). MB-PDT had a synergistic effect when used in combination with polyhexamethylene biguanide (PHMB) or amphotericin B, but not with voriconazole.

CONCLUSIONS. MB-PDT is effective against Acanthamoeba in vitro and has synergistic effects with PHMB and amphotericin B. (Invest Ophthalmol Vis Sci. 2012;53:6305–6313) DOI:10.1167/iovs.12-9828

Acanthamoeba keratitis (AK) is a severe and sight-threatening ocular infection, which usually occurs in the context of soft contact lens (SCL) wear or trauma. Acanthamoeba are morphologically classified as trophozoites, which can take up nutrition and proliferate, and dormant cysts, which resist insults from high temperatures, dryness, and drugs. Acanthamoeba can change into trophozoite or cyst form to adjust to various environments. Treatment is usually carried out with a combination of antifungal medications, primarily biguanides such as polyhexamethylene biguanide (PHMB) and chlorhexidine, and diamidines such as propamidine isethionate and hexamidine. However, these medications can often be toxic to the cornea. In addition to medication, sometimes epithelial debridement is performed to make a histologic diagnosis of amoebal infection, or to physically remove amoeba and increase penetration of medication into the tissue. However, even when these therapies are combined, many cases are resistant to treatment, and ultimately 5% to 30% of cases are reported to require therapeutic or optical corneal transplant. Due to the rapid emergence of cases, new therapies or prophylaxis regimens for AK are urgently needed.

Riboflavin and UV light–induced cross-linking have been reported to constitute a mechanical treatment for corneal infection, which can be expected to produce immediate effects. Photodynamic therapy (PDT), performed with a light source and a photosensitizer (PS), has garnered attention as another form of antimicrobial therapy. PDT takes advantage of the capacity of PS to accumulate in certain target cells. After the PS is administered and irradiated by light of a certain wavelength, it is excited from the ground state through the excited singlet state to a triplet state. In the presence of oxygen, the PS undergoes reactions that produce reactive oxygen species and induce cell damage via oxidative stress. Since the PS localizes to certain cells, only target cells in the irradiated area are damaged. Various types of PS are used in PDT, but methylene blue (MB; wavelength of maximum absorption: 600–660 nm), a phenothiazinium PS, has been particularly widely used in histology for more than 100 years. MB has the potential to treat a variety of cancerous and noncancerous diseases, with low toxicity and no side effects. Phenothiazinium PS is known to exhibit antimicrobial effects after exposure to light, and many reports indicate that PDT is effective against bacteria, viruses, and protozoa. However, there are no reports of using PDT with MB (MB-PDT) to treat acanthamoeba infection, and it is not clear whether PDT is effective against amoeba or how MB acts on the organism.

The aim of the present study was to investigate the in vitro amoebicidal effect of two therapies: MB-PDT; and riboflavin and ultraviolet A cross-linking. We also sought to determine whether the effect of MB-PDT is retained or augmented when it is performed in combination with conventional antiamoebic agents.

MATERIALS AND METHODS

Organisms and Culture

All studies were performed with Acanthamoeba castellanii strain ATCC 50370 (American Type Culture Collection, Manassas, VA), which...
was originally isolated from a case of AK. Trophozoites were axenically grown in peptone-yeast extract-glucose (PYG) medium at 25°C in a tissue-culture flask (Becton Dickinson, Tokyo, Japan). Encystment was induced by transferring the trophozoites from the PYG medium to Nef’s constant-pH encystment medium22 and incubating the trophozoites for at least 2 weeks at 25°C. Another pathogenic strain of Acanthamoeba castellanii (ATCC 30868) and four clinical isolates of Acanthamoeba from patients diagnosed with AK in the Ehime Institute of Ophthalmology in 2009 and 2010 were used for comparison purposes.

Preparation of Photosensitizer and Irradiation Source

Methylen blue (MB) (Sigma-Aldrich, Inc., St. Louis, MO) was dissolved in deionized water to give a 10 mM stock solution and stored in the dark until use. It was then diluted by the appropriate volume to obtain the test solutions and filter-sterilized using a 0.22-μm pore size membrane filter. The light source used was a halogen lamp (noncoherent LC-122A; LumaCare, Newport Beach, CA), equipped with a band-pass filter probe to isolate the 650- to 670-nm wavelength interval. The fluorescence rate was measured by a power meter device with a thermal sensor (10A; Ophir Optronics Ltd., Jerusalem, Israel) and display (FieldMate laser power meter; Coherent Inc., Santa Clara, CA).

Acanthamoeba Respiratory Activity Assay

To estimate the efficacy of treatment, the 5-cyano-2,3-dihydro-1,4-naphthoquinone chloride (CTC) biocidal assay was performed as described previously23 to assess the respiratory activity of Acanthamoeba. Briefly, reagents from the bacterial staining/CTC staining kit (Bacstain-CTC Rapid Staining Kit; Dojindo Laboratories, Kumamoto, Japan) were added to samples, and cells were incubated for 30 minutes at 25°C according to the manufacturer's recommendations. Each amoeba suspension was tested in quintuplicate, and fluorescence intensity was measured with a fluorescence microplate reader (FlexStation 3; Molecular Devices, Sunnyvale, CA; excitation, 480 nm; emission, 630 nm). Sodium azide was used to inhibit respiration in samples used as negative controls.

Cell Photosensitization Studies

The phototoxicity of MB toward trophozoites and cysts was examined as a function of the MB concentration. Trophozoites or cysts were collected from a flask, washed with PBS, and centrifuged at 150g for 10 minutes, resulting in a pellet of 4 × 106 organisms. The pellets were resuspended in various concentrations of MB solution (0, 0.05, 0.1, 0.25, and 0.5 mM) and incubated for 10 minutes in the dark. After incubation, the organisms were washed and resuspended in 4 mL of PBS. The amoeba suspension was divided into two portions, and each 2 mL portion was transferred to a 35-mm petri dish. The cover of each dish was removed and a probe was placed above the dish to measure light intensity. One dish was irradiated with light from a light source (365-nm wavelength; UVL-56 handheld UV Lamp; UVP Inc., Upland, CA) for 1 hour at an intensity of 3 mW/cm2 as measured.

The effect of light irradiation dosage on Acanthamoeba was also studied. Trophozoites (4 × 106 organisms) were incubated in 0.5 mM MB solution for 10 minutes following the experimental protocol described above. The cells were subsequently transferred to a 35-mm petri dish and were irradiated with light for either 5, 10, 20, or 30 minutes, resulting in light doses of 1.8, 3.6, 7.2, and 10.8 J/cm2, respectively. To evaluate the effect of pulse irradiation, samples were irradiated with 5- or 10-minute pulses of light at 10-minute intervals, for a total of 30 minutes of irradiation and a total light dose of 10.8 J/cm2. All experiments were carried out with a control group kept in the dark as described earlier.

Microscopic observations of samples before and after PDT were made with a phase-contrast microscope at ×20 magnification without fixation. Subconfluent cultures of trophozoites were incubated in 0.5 mM MB solution for 10 minutes, and gently washed with PBS twice, taking care to keep the trophozoites from detaching from the bottom of the dish. Afterward, the dishes were refilled with PBS. Trophozoites were irradiated for 30 minutes and, immediately after irradiation, the dish was placed in a dark area for 150 minutes. Photographs were obtained with a differential interference contrast device (Zeiss Axio Observer Z1; Carl Zeiss MicroImaging GmbH, Jena, Germany) before irradiation, after 10, 20, and 30 minutes of irradiation, and 150 minutes after the completion of irradiation. For the purpose of comparison, control groups were also observed after being treated under the following three conditions: incubation in PBS and no irradiation (L–M–), incubation in PBS and irradiation (L–M+), incubation in MB, and no irradiation (L–M–). Finally, cell viability and survival rate at 150 minutes after the completion of irradiation (or nonirradiation) were determined by trypan blue exclusion24,25 and the culture-dependent biocidal assay.26,27 Respectively. The survival rates of PDT-treated cysts of Acanthamoeba, as determined by the culture-dependent biocidal assay. To check for morphologic changes in trophozoites after PDT, transmission electron microscopy (TEM) was performed using standard procedures described elsewhere.28,29 Ultrathin sections (<60–80 nm) were double-stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (JEM 1230; JEOL Ltd., Tokyo, Japan) at 100 kV.

Combined Antiamoebic Drug and Photodynamic Therapy

PHMB, amphotericin B, and voriconazole were used as antiamoebic drugs to determine whether antiamoebic drugs have a synergistic effect with PDT. PHMB was diluted in 1/4 Ringer’s solution to obtain test solutions with final concentrations of 1.25, 5, 10, 25, 50, and 100 ppm. Amphotericin B (Fungizone; Bristol-Myers K.K., Tokyo, Japan) was reconstituted in sterile Millipore-filtered water and diluted to concentrations of 100, 200, 400, 800, and 1600 μg/mL. Voriconazole (Vfend; Pfizer, Tokyo, Japan) was reconstituted in sterile Millipore-filtered water and diluted to concentrations of 40, 100, 400, 1000, 4000, and 10,000 μg/mL. To evaluate the effect of each antiamoebic drug alone, 4 × 108 trophozoites were incubated in different concentrations of each antiamoebic drug for 60 minutes. Then the cells were washed and the CTC biocidal assay was performed. Based on the results of preliminary experiments with MB and antiamoebic drug monotherapy, sublethal concentrations of each solution were selected to test in combination against trophozoites, to evaluate for a synergistic effect. To assess the effect of combination therapy, 4 × 108 trophozoites were incubated with each antiamoebic drug for 60 minutes, washed with PBS twice, incubated with MB solution in the dark for 10 minutes, and irradiated as described above. After irradiation at 10.8 J/cm2 (or nonirradiation), cells were collected from dishes and the CTC biocidal assay was performed.

Riboflavin and Ultraviolet A Light Treatment

Trophozoites (4 × 106 organisms) were incubated for 60 minutes in the dark in a riboflavin solution with 20% Dextran 500 (Medio-Cross; Medio-Haus Medizinprodukte GmbH, Rostock, Germany). Afterward, the cells were washed and pellets were resuspended in 4 mL of PBS. The suspension was divided into two portions, and each 2 mL portion was transferred into a 35-mm petri dish. The cover of the dish was removed and the suspension was exposed to an Ultraviolet A (UVA) light source (365-nm wavelength; UVL-56 handheld UV Lamp; UVP Inc., Upland, CA) for 1 hour at an intensity of 3 mW/cm2 as measured.
by a UV light meter (J-221, 365 nm; UVP Inc.). After irradiation, samples were collected and the CTC biocidal assay was performed. The CTC biocidal assay was also performed on control samples treated under the following conditions: PBS incubation only, UVA exposure only, and riboflavin solution incubation only.

**Statistical Analyses**

In the cell photosensitization studies for trophozoites and cysts, the light and dark groups were compared using the Student’s t-test, assuming equal variances. One-way ANOVA was performed to determine whether there were significant differences between the different test conditions. If a significant difference was found between the groups as a whole, Dunnett’s tests were performed to determine where these differences occurred. \( P < 0.05 \) was used to indicate statistical significance.

**RESULTS**

**Effect of Photodynamic Therapy on Trophozoites and Cysts**

The light irradiation–mediated effect of PDT was evaluated at various concentrations of MB (Fig. 1A). First, we confirmed that there was no significant decrease in trophozoite respiratory activity due only to light irradiation (10.8 J/cm\(^2\)) by comparing trophozoites incubated in PBS and irradiated (PBS light group) with those not irradiated (PBS dark group). Next, we evaluated the concentration-dependent effect of MB with and without light irradiation. In the dark groups, there was no difference between the respiratory activity of trophozoites incubated at any MB concentration below 0.5 mM and that of the PBS dark control group. In the light groups, PDT was not effective when carried out at concentrations of MB of 0.05 and 0.1 mM. However, at higher concentrations of MB, the efficacy of PDT was found to increase in a concentration-dependent manner. When trophozoites were incubated in 0.5 mM MB for 10 minutes, and subsequently irradiated for 5 (1.8 J/cm\(^2\)), 10 (3.6 J/cm\(^2\)), 20 (7.2 J/cm\(^2\)), or 30 minutes (10.8 J/cm\(^2\)), respiratory activity was found to be significantly less than that of the dark control group (Fig. 1B). In addition, when trophozoites were exposed to 5- or 10-minute pulses of light at 10-minute intervals (total irradiation time 30 minutes), the effect of PDT was found to be equally as strong as that in the group that received 30 minutes of continuous irradiation. With regard to cysts (Fig. 1C), a significant difference in respiratory activity was noted between the dark and light groups at all tested concentrations of MB.

Figure 2A shows the condition of trophozoites before and after irradiation following incubation in MB. Untreated organisms (L–M– group), organisms only irradiated (L–M+ group), and organisms only incubated in MB (L+M– group) were used as control groups. Before irradiation, trophozoites in all groups adhered to the bottom of the dish, and no morphologic differences were observed between the groups (Fig. 2A, a, b, c, d). In the control groups, almost no change was noted over time (Fig. 2A, e, f, g). In contrast, trophozoites in the PDT group began to become round (data not shown) and detach from the bottom of the dish after approximately 10 minutes of irradiation, and after 30 minutes, almost all of the trophozoites were detached and floating in the medium (Fig. 2A, h). Figure 2B shows the percentage of viable cells in each group identified by trypan blue staining 150 minutes after irradiation. In the L–M–, L–M+, and L+M– groups, 94.1%, 97.0%, and 89.8% of trophozoites failed to stain with trypan blue, respectively, whereas the cell viability for the PDT group was 0.6%, substantially lower than the controls. When the trophozoite survival rate was assessed by a conventional culture-dependent biocidal assay, the survival rates were 111.4%, 103.1%, 111.4%, and 8.5% for the abovementioned groups (Fig. 2C), showing a trend similar to that obtained by trypan blue staining. Cyst survival rates were 103.2%, 111.4%, 96.8%, and 55.4% for the abovementioned groups (Fig. 2D).

**Effect of Photodynamic Therapy on Clinical Isolates**

The effects of MB incubation, light irradiation, and MB-PDT were also tested using several additional amoebic strains, including two types of *Acanthamoeba castellanii* from ATCC and four clinical isolates from patients with infectious keratitis. Respiratory activity in each strain after treatment under each condition is shown in the Table. MB-PDT induced significant loss of respiratory activity, with mean values ranging from 0% to 27% in the six strains.

**Effect of Combined Photodynamic Therapy and Antiamoebic Drug Therapy**

In this portion of the study, PDT was conducted under the following conditions. Trophozoites were irradiated at 10.8 J/cm\(^2\) after incubation for 10 minutes in 0.1 mM MB, the MB concentration at which trophozoite respiratory activity was not affected in previous experiments. The efficacy of each individual antiamoebic drug was evaluated with the CTC biocidal assay in a preliminary experiment (data not shown), and the effects of combination therapies were investigated using concentrations of each drug that were judged to produce a weak effect in the preliminary experiment (e.g., 1 and 2.5 ppm PHMB, 100 and 200 µg/mL amphotericin B, 400 and 1000 µg/mL voriconazole). PHMB and amphotericin B were found to have a synergistic effect with MB-PDT. Combination therapy of PHMB and 0.1 mM MB without irradiation reduced trophozoite respiratory activity significantly, compared with single-agent therapy with PHMB. When irradiation was added to this treatment regimen, respiratory activity significantly decreased to 2.9% and 2.2% of the control for groups treated with 1 and 2.5 ppm PHMB, respectively (Fig. 4A). A similar trend was noted with amphotericin B. When amphotericin B–treated trophozoites were incubated in MB, no change was observed in respiratory activity. In contrast, when irradiation was added, respiratory activity decreased to 2.8% and 3.3% of the control for amphotericin B concentrations of 100 and 200 µg/mL, respectively (Fig. 4B). No synergistic effect was observed with MB-PDT and voriconazole (Fig. 4C).

**Effect of Riboflavin/UVA Combination Therapy on Trophozoites**

Figure 5 shows the effect of combination riboflavin and UVA irradiation therapy on trophozoite respiratory activity. The respiratory activity of trophozoites irradiated with UVA for 60 minutes at an intensity of 3 mW/cm\(^2\) was 111.7% of that of the
FIGURE 1. Average amoebicidal effects of photodynamic therapy. Irradiated groups were incubated in MB for 10 minutes, washed with PBS three times, and then exposed to light from a halogen lamp (660 nm). A value of 100% was assigned to the respiratory activity of control untreated cells. (A) Trophozoite respiratory activity as a function of MB concentration with or without light irradiation for 30 minutes (total light dosage of 10.8 J/cm²). *P < 0.05, compared with the dark cells. (B) Effect of irradiation time on phototoxicity of 0.5 mM MB against trophozoites. Cells were irradiated with light for 5, 10, 20, and 30 minutes, resulting in light dosages of 1.8, 3.6, 7.2, and 10.8 J/cm², respectively. Additional samples were exposed to 5- or 10-minute pulses of light at 10-minute intervals, for a total of 30 minutes of irradiation and a light dosage of 10.8 J/cm². The respiratory activity in all irradiated groups was significantly different from that of the dark group (*P < 0.05). (C) Cyst respiratory activity as a function of MB concentration with or without light irradiation for 30 minutes (total light dosage of 10.8 J/cm²). *P < 0.05, compared with the dark cells.
FIGURE 2. (A) Light microscope (phase-contrast) images of trophozoites before light exposure (a-d) and 150 minutes after light exposure (total light dosage of 10.8 J/cm²) (e-h). (a, e) L–M– group; (b, f) L+M– group; (c, g) L–M+ group; (d, h) L+M+ group. Bar: 50 μm. (B) Comparison of cell viability after each treatment regimen. Cell viability was assessed via trypan blue exclusion performed 150 minutes after the completion of light irradiation. (C) Comparison of survival rate after each treatment regimen. Survival rate was assessed by culture-dependent biocidal assay. *P < 0.01, compared with the dark cells. (D) Survival rate for cysts after treatment with 0.5 mM MB-PDT. *P < 0.05, compared with the dark cells.
untreated control organisms. When trophozoites were incubated in 0.1% riboflavin for 60 minutes without UVA irradiation, the respiratory rate was 104.0% of the control. The respiratory rate was 91.9% of the control when trophozoites were treated with a combination of 0.1% riboflavin and UVA irradiation. No significant difference was noted between any of these treatment regimens and the untreated control group.

**DISCUSSION**

The work outlined here is directed toward the development of PDT as a novel method for the treatment of AK. The findings described in the present study indicate that the phenothiazinium dye MB has a photodynamic effect on *Acanthamoeba* when the microorganism is in both the trophozoitic and cystic stages.

A few experimental investigations have demonstrated that PDT conducted with tetracationic phthalocyanine (RLP068) or perylenequinonoids (hypocrellin B) can effectively kill *Acanthamoeba*, but no previous reports have addressed important issues related to the clinical application of PDT, such as necessary irradiation time, comparative efficacy against different clinical strains, synergistic effects with antiamoebic drugs, or comparison with other treatments that use a light source.

Our findings suggest that the CTC biocidal assay can be used as an alternative method to assay for living *Acanthamoeba* organisms instead of the conventional culture-dependent assay using the most probable number (MPN) method or the Spearman-Karber method. The culture-dependent biocidal assay requires 1 to 3 weeks of cultivation of trophozoites and cysts, respectively, to detect surviving *Acanthamoeba*. In contrast, the CTC biocidal assay can be carried out in only a few hours for trophozoites (30 minutes for staining and 30 minutes for fixation after the treatment), and in a day for cysts, which require preincubation for 16 hours before CTC staining. In addition, in both our MB-PDT experiments and a previous study that evaluated treatment of *Acanthamoeba* with PHMB and SCL disinfectants, the respiratory activity determined by the CTC biocidal assay was confirmed to be significantly correlated with the survival rate determined by the culture-dependent biocidal assay.

We chose to use MB as the PS in our PDT experiment because MB has low toxicity. Shih and Huang reported that no complications were seen after instillation of 1% (approximately 31 mM) MB on denuded cornea four times per day for 3 days. Intact epithelia prevent MB from staining intact rabbit cornea, so debridement is thought to be necessary for local eye drop administration of MB. In the actual treatment of AK, epithelial debridement has many other benefits, including facilitation of histologic diagnosis of amoebal infection, physical removal of amoeba, and penetration of medication. Since MB has low toxicity, it can be used in combination with epithelial debridement without being problematic, especially if debridement is carried out locally in the area of the lesion.

Another reason we chose MB is that MB is positively charged, enabling it to rapidly bind to the negatively charged cell membrane, mitochondrial membrane, or nucleic acids. Since mitochondrial membranes and nucleic acids have a stronger negative charge than that of the cell membrane, if MB is taken into the cell, it is likely to localize in mitochondria, lysosomes, and areas with nucleic acids. Targeting mitochondria is an important research subject in PDT; since it is thought that damaging mitochondria may induce the apoptotic cascade. However, in our experiment, mitochondria were not found to be decreased in the MB-PDT group compared with the other group. Instead, our TEM findings showed a notable disappearance of nucleic acids in most trophozoites that underwent MB-PDT. From these findings, it appears that in the present experiment, the direct effect of MB-PDT on nucleic acids may play a more important role in the amoebicidal mechanism of MB-PDT than apoptosis mediated by mitochondrial damage. This may be the case because MB is hydrophilic and an ideal nucleic acid intercalator due to its small size (MW 319.85 g/mol) and linear tricyclic heteroaromatic structure.

In the present experiment, there was no significant difference between the efficacy of 30 minutes of continuous irradiation and 30 minutes of irradiation divided into three pulses. These results suggest that the effect of PDT on...
Acanthamoeba respiratory activity is dependent on the dose of radiation. In previous reports, PDT has been found to have a similar radiation dose-dependent effect on other pathogens besides acanthamoeba.\textsuperscript{17–20} In other words, the efficacy of PDT is thought to increase in proportion to light intensity and exposure time.

We found that conventionally used medications and MB-PDT have an additive or synergistic effect against trophozoites. This suggests that MB-PDT could be a valuable adjuvant to antimicrobial therapy in AK cases that show little or no improvement with conventional antiamoebal treatment. Both PHMB and amphotericin B produced a particularly notable synergistic effect with MB-PDT against trophozoites. PHMB induces changes in cell membrane permeability, leading to potassium efflux and eventual loss of membrane function and cell death.\textsuperscript{41}

\begin{figure}[h]
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\caption{Combined effect of MB-PDT (incubation in 0.1 mM MB and irradiation at a dose of 10.8 J/cm\(^2\)) and antimicrobial drug pretreatment at various concentrations. PHMB (A) and amphotericin B (B) were found to have a synergistic effect with MB-PDT, whereas MB-PDT and voriconazole (C) did not show a synergistic effect compared with the antimicrobial drug control \(P < 0.05\). \(*P < 0.05\).}
\end{figure}

As with other polyene antifungals, amphotericin B
is believed to interact with membrane sterols and produce an aggregate that forms a transmembrane channel. The synergistic effects of MB and medication are brought about because MB can leak into the cell more quickly after prior damage to the cell membrane.

In contrast, voriconazole requires a longer period of time to exert a membrane-damaging effect compared with PHMB and amphotericin B, and thus combinations involving voriconazole did not show a synergistic effect. Since AK treatment generally takes a long time, medication toxicity can occasionally become a problem. In situations where two medications exert a synergistic effect, toxicity can be avoided by lowering each medication dosage below the concentration usually used in single-agent therapy. In addition, it has been reported that organisms can easily become resistant to low-dose single-drug antiamoebic therapy, so by using multiple-agent therapy, the emergence of resistance can be prevented.

Cross-linking induced by riboflavin–UVA combination therapy, which is often used to treat keratoconus, works similarly to MB-PDT by producing reactive oxygen species. Recently, a series of reports have been published regarding the promising clinical applications of cross-linking for corneal infections. Khan et al. contend that cross-linking is useful for AK. However, our in vitro experimental results suggest that cross-linking is markedly less effective than MB-PDT against trophozoites. Similarly, Del Buey et al. concluded that a single dose (30 or 60 minutes) of cross-linking cannot achieve eradication of the two different Acanthamoeba strains examined. However, their evaluation method did not quantify reductions in the population of viable amoeba; it detected only the presence of amoeba, and their growth and movement in the agar media. In this respect, the CTC biocidal assay is advantageous because it enables quantitative comparison. Our study using the CTC biocidal assay shows that MB-PDT is more effective than riboflavin–UVA combination therapy.

We suggest that MB-PDT has the potential to provide a valuable adjuvant to antimicrobial therapy in AK when combined with antiamoebal agents. Since the cornea is an organ that is exposed to the outside world, local administration of medication and irradiation can be carried out easily, and for this reason, AK therapy appears to be a feasible application of PDT. Furthermore, we examined the toxicity of PDT treatment in vivo using C57BL/6 mouse corneas with epithelial defects, and found that MB-PDT did not cause any appreciable damage to the cornea (see Supplementary Fig. S1).
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