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Original Article

5-Aminolevulinic acid induced photodynamic inactivation on *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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ABSTRACT

The aim of the present study was to develop a simple and fast screening technique to directly evaluate the bactericidal effects of 5-aminolevulinic acid (ALA)-mediated photodynamic inactivation (PDI) and to determine the optimal antibacterial conditions of ALA concentrations and the total dosage of light *in vitro*. The effects of PDI on *Staphylococcus aureus* and *Pseudomonas aeruginosa* in the presence of various concentrations of ALA (1.0 mM, 2.5 mM, 5.0 mM, 10.0 mM) were examined. All bacterial strains were exponentially grown in the culture medium at room temperature in the dark for 60 minutes and subsequently irradiated with 630 ± 5 nm using a light-emitting diode (LED) red light device for accumulating the light doses up to 216 J/cm^2. Both bacterial species were susceptible to the ALA-induced PDI. Photosensitization using 1.0 mM ALA with 162 J/cm^2 light dose was able to completely reduce the viable counts of *S. aureus*. A significant decrease in the bacterial viabilities was observed for *P. aeruginosa*, where 5.0 mM ALA was photosensitized by accumulating the light dose of 162 J/cm^2. We demonstrated that the use of microplate-based assays—by measuring the apparent optical density of bacterial colonies at 595 nm—was able to provide a simple and reliable approach for quickly choosing the parameters of ALA-mediated PDI in the cell suspensions.

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1. Introduction

The widespread inappropriate use of antibiotics has resulted in multiresistant bacterial strains and increased rates of infection [1,2]. This health care problem is therefore particularly urgent because there is a clear need for a more effective anti-infective strategy against these organisms. Many of the new antibiotics are more potent, but they also increase the risk of systemic toxicity. The versatility and potency of photodynamic therapy (PDT) may be an interesting alternative choice against many types of microorganisms and the lack of resistance with repeated use [3]. Basically, PDT is the result of the use of three autonomously nonactive elements in combination: (1) a nontoxic photoactive molecule called a photosensitizer (PS); (2) light of the appropriate wavelength to excite the PS; and finally, (3) oxygen, which is transformed into the highly reactive singlet oxygen species upon energy transfer from the light-activated PS.

The photodynamic effect uses nontoxic dyes or PS in combination with exposure to harmless visible light in the presence of oxygen to induce the generation of highly reactive, cytotoxic reactive oxygen species, causing selective photodamage to tumor tissues or leading to localized cell death [4]. It provides the following advantages for treatment of microbial infections: (1) broad light spectrum of action; (2) efficient inactivation of antibiotic-resistant strains; (3) low mutagenic potential; and (4) less likelihood of inducing photoresistant cells [5,6]. Bacterial organisms such as Gram-positive bacteria can be killed by photodynamic inactivation (PDI) *in vitro* with exogenous PS such as porphyrins. The complex outer membrane of Gram-negative bacteria acts as a barrier that hinders the PS to transport through the cell membranes; hence, Gram-negative bacteria appear to be less sensitive to the lethal action of PDI with exogenously supplied porphyrins [7–9].

5-Aminolevulinic acid (ALA) is a naturally occurring intermediate in the hemesynthesis pathway [10]. It is a precursor of porphyrins that can be biosynthesized in nearly all aerobic cells in mammals. The first topical application of ALA in the treatment of basal cell carcinoma was reported in 1990 [11]; since then, the clinical use of ALA-PDT continues to grow. ALA-PDT has been widely studied and marketed around the world [12], and the methyl-ester derivative of ALA has been approved for the treatment of basal cell carcinoma and actinic keratosis [13]. In addition, the European Medicines Agency has approved the hexyl-ester derivative of ALA for diagnostic application in endoscopic photodynamic detection of bladder cancer and ALA for intraoperative photodiagnosis of residual malignant glioma [14].

The reasons why ALA was extensively used in the field of PDT can be summarized as follows: (1) ALA is the only PDT agent that is a biochemical precursor of a PS, which is naturally produced by the body, and alone shows low dark toxicity to cells; (2) the topical delivery of ALA does not induce any prolonged photosensitivity reactions, because the drug can be selectively applied in areas to be treated; (3) endogenously produced protoporphyrin IX is rapidly cleared from the body (24–48 hours), because it has a natural clearance mechanism; and (4) the short time interval (1–8 hours, depending on the mode of administration) needed between the administration of ALA and the maximal accumulation of protoporphyrin IX in target tissues makes ALA attractive for patients.

Furthermore, ALA has been shown to have considerable photobactericidal activity. Compared to exogenously administered hydrophobic porphyrin derivatives, ALA is highly water soluble and may enter the intracellular compartment of Gram-negative bacteria through the hydrophilic pores of its outer membrane [15]. Treatment with exogenous ALA could effectively accumulate considerable amounts of photoactive porphyrins (PAPs) within the targeted cells [16]. Under the irradiation of the appropriate wavelength of light, the accumulated porphyrins will induce PDI to destroy the cells [17]. Recently, a few reports showed that ALA could induce PDI effectively against various kinds of bacterial strains such as Gram-positive *Staphylococcus aureus* and Gram-negative bacteria *Pseudomonas aeruginosa* and *Escherichia coli* [18–24].

Although PDI of bacteria has been known for more than 100 years [25], its use for treatment of infections has not been extensively developed [26]. This may be partly attributable to the lack of a standardized and reliable *in vitro* screening method to evaluate the antibacterial efficacy of PDT. Our study aimed to assess the effectiveness of ALA-mediated PDI on *S. aureus* and *P. aeruginosa* by directly judging the apparent optical density (OD) caused by light scattering of colonies and further to determine the optimal antibacterial conditions of ALA doses and light exposure *in vitro*. We have developed a more economic and rapid *in vitro* screening technique to evaluate the antimicrobial activity of ALA-PDT in contrast to the traditional antibacterial susceptibility testing.

2. Materials and methods

2.1. Materials

ALA and phosphate buffer solution (PBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nutrient broth medium (BD 234000) and nutrient agar medium (BD 4311472) were purchased from Difco (Detroit, MI, USA).

2.2. Preparation of ALA solution

A stock solution of 100 mM ALA was prepared by dissolving ALA in 0.1 M PBS (pH 7.4) prior to the experiment. The ALA stock and diluted solutions were used within 2 hours after the preparation to ensure its stability.

2.3. Bacterial strains and growth conditions

This study was conducted with a Gram-negative strain (*P. aeruginosa*; American Type Culture Collection Strain 27853) and a Gram-positive strain (*S. aureus*; American Type Culture Collection Strain 29213) purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). The strains were grown in nutrient broth separately for 24 hours at 37°C in a humidified atmosphere of 95% air and 5% CO_2 controlled by a low-temperature incubator (LE-509; YIH DER Instruments, Taipei, Taiwan). The broth cultures were then spread on nutrient agar medium and then incubated at 37°C

for 24 hours. This incubation process was repeated for three times to produce the bacterial strains containing approximately 10^8 colony-forming units (CFU)/mL.

2.4. PDI of bacterial cells

For PDI, bacterial cells in the broth cultures were centrifuged, washed three times with PBS, and then suspended in PBS to obtain a cell suspension about 10^8 CFU/mL. Aliquots of suspensions (0.1 mL) were transferred into 96-well plates, and then 0.1 mL of different concentrations of ALA solution (0–10 mM) was added. Samples were incubated for 60 minutes in the dark and then irradiated at room temperature (ca. 25°C). The light source used for ALA irradiation consisted of a high-power light-emitting diode (LED) array with the wavelength centered at 635 ± 5 nm, with an irradiance set as required light doses [27]. The irradiated and nonirradiated bacterial cells (10 mL) were serially diluted 10-fold with PBS and incubated for an additional 18 hours at 37°C.

The apparent ODs caused by light scattering of colonies were measured by using a Microplate Autoreader (EL311; Bio-Tek Instruments, Winooski, VT, USA) at 595 nm; the OD values were subsequently calibrated with the CFU obtained by plate counts. Afterward, the CFU can be easily calculated from the calibration curve (see Fig. 1A for *S. aureus* and Fig. 1B for *P. aeruginosa*) by interpolating the measured OD value. All results are expressed as the mean ± standard deviation. Differences between two means were assessed for significance by the two-tailed Student *t* test, and a $p < 0.05$ was considered statistically significant.

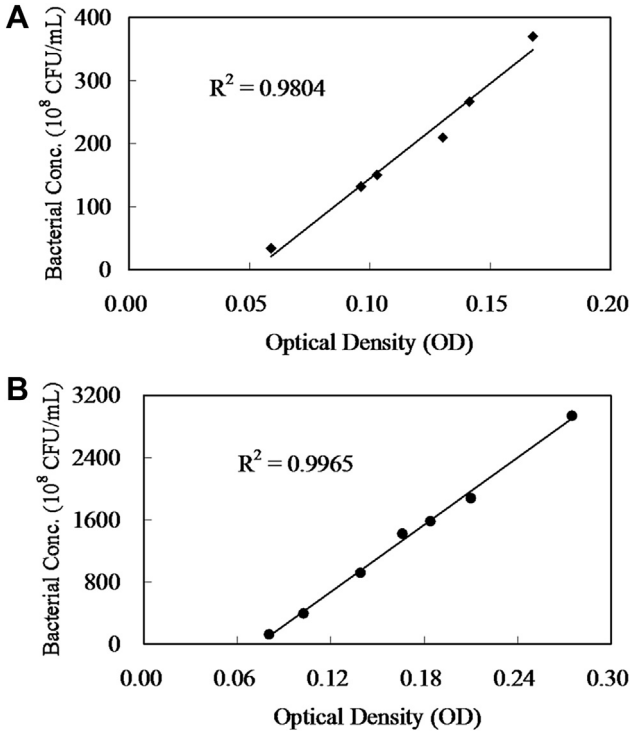

Fig. 1 – Calibration curves of optical density (595 nm) versus bacterial concentration: (A) *Staphylococcus aureus* and (B) *Pseudomonas aeruginosa*. All data are expressed as the mean ± standard deviation.

2.5. Bacterial cell survival assay

The numbers of CFU of a bacterial suspension were determined by plating appropriate dilutions (from 10^{-1} to 10^{-5}) on trypticase soy broth agar plates. The survival fraction was calculated as N_{PDI}/N_0, where N_{PDI} is the number of CFU per milliliter after PDI and N_0 is the number of CFU per milliliter in the initial sample. The dark toxicity of the substrates, defined as the intrinsic toxicity of the compounds in the absence of light, was monitored by evaluating the survival fraction of incubated but nonilluminated bacterial samples and was calculated as N_{dark}/N_0, where N_{dark} is the number of CFU per milliliter of the nonilluminated samples. The results were expressed as mean values ($n = 3$) with their standard deviations.

3. Results and discussion

3.1. Dark toxicity of ALA on the bacterial strains

Preliminary experiments were carried out in order to confirm the relationship between CFU value and OD. As shown in Fig. 1, significant positive correlations between the colonies of both bacterial strains and the measured light responses were observed ($R^2 = 0.9804$ for *S. aureus* in Fig. 1A; $R^2 = 0.9965$ for *P. aeruginosa* in Fig. 1B). Therefore, the use of OD to estimate CFU value in a suspension was proven to be a simple and applicable method for the following experiments.

The bacterial cultures (*S. aureus* and *P. aeruginosa*) were incubated in the dark for 60 minutes with ALA at various concentrations in order to evaluate the dark toxicity of ALA. For *S. aureus*, the trends of gradually increasing ODs with time were consistent with the control group (incubated without ALA, data not shown); the bacterial survival of *S. aureus* was not affected by ALA when incubated in the dark, indicating that ALA induced no dark toxicity on *S. aureus* cells. On the contrary, the number of surviving colonies of *P. aeruginosa* (calculated by interpolating the measured OD value into Fig. 1B) was partly inhibited by higher ALA concentrations (5.0 mM and 10.0 mM), whereas negligible reductions in the surviving colonies were found in the conditions of 1.0 mM and 2.5 mM ALA (data not shown). The growth inhibition of *P. aeruginosa* observed here is possibly due to higher concentrations of ALA, which leads to the increasing acidification of the culture. A lower pH culture medium was reported to exhibit a rapid bactericidal effect against Gram-negative bacteria such as *P. aeruginosa*, but this effect was not observed on Gram-positive bacteria [28]. In addition, the finding seemed to suggest that pretreatment with ALA is likely to strengthen the bactericidal effects on *P. aeruginosa*.

3.2. PDI against S. aureus

The effects of photoirradiation doses on *S. aureus* with various ALA concentrations were studied. As shown in Fig. 2A, when *S. aureus* was incubated with different concentrations of ALA and exposed to 216 J/cm^2 of red light (irradiated for 120 minutes), a significant reduction in the surviving cells can be achieved regardless of ALA concentrations, even in the

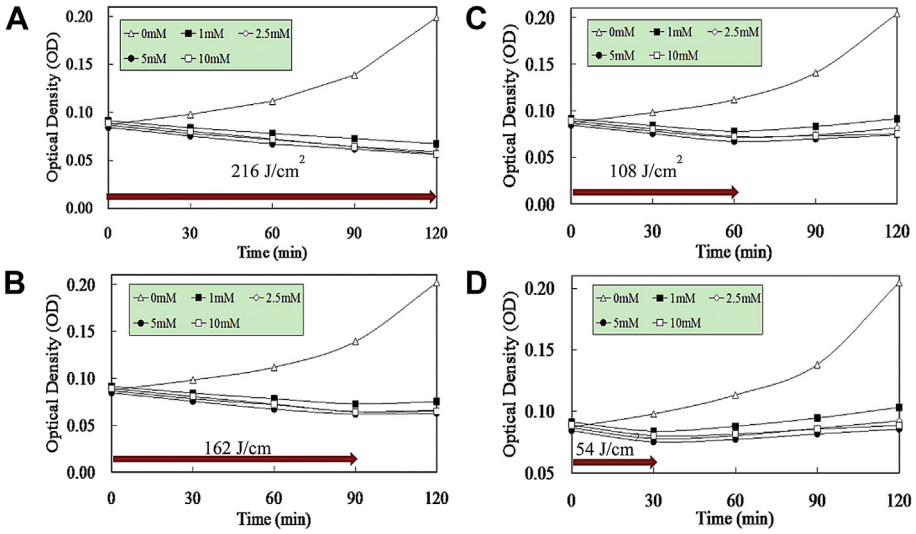

Fig. 2 – 5-Aminolevulinic acid (ALA)-induced photodynamic inactivation against *Staphylococcus aureus* in the presence of 0 mM, 1.0 mM, 2.5 mM, 5.0 mM, and 10.0 mM ALA. Photoirradiation time was set at (A) 120 minutes, (B) 90 minutes, (C) 60 minutes, and (D) 30 minutes for accumulating light doses of 216 J/cm^2, 162 J/cm^2, 108 J/cm^2, and 54 J/cm^2, respectively. All data are expressed as the mean ± standard deviation.

presence of a low ALA concentration (1.0 mM). It demonstrated that PDI against *S. aureus* was induced successfully by ALA at the condition. As with reducing the photoirradiation dose to 162 J/cm^2 (Fig. 2B), a significant reduction in the surviving cells was also observed after 90 minutes of irradiation (treated with 1.0 mM ALA), and no further proliferations could be detected thereafter in all experimental groups. Slightly different results were found in Fig. 2C and 2D (108 J/cm^2 and 54 J/cm^2 light doses, respectively), wherein the cell proliferations of irradiated samples were suppressed during photoirradiation, whereas the bacterial survival of *S. aureus* incubated with various ALA concentrations gradually grew once the light was removed. To summarize the results, an economic and time-saving formulation to effectively induce PDI against *S. aureus* could be obtained through incubation with 1.0 mM of ALA and exposure to 162 J/cm^2 of light dose.

3.3. *PDI against P. aeruginosa*

When the period of photoirradiation was increased from 30 to 120 minutes, the bacterial killing of *P. aeruginosa* gradually increased as shown in Fig. 3. In the presence of 1.0 mM ALA, the reduction in surviving cells (taken from interpolating the OD values to the calibration curve) was estimated to be about 2.3 log, 3.3 log, 4.0 log, and 4.7 log, whereas irradiating time was set as 30 minutes, 60 minutes, 90 minutes, and

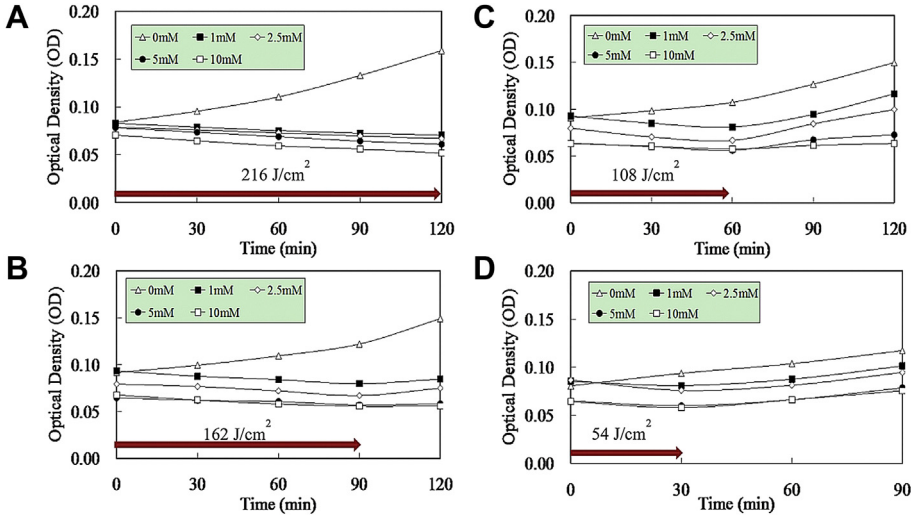

Fig. 3 – 5-Aminolevulinic acid (ALA)-induced photodynamic inactivation against *Pseudomonas aeruginosa* in the presence of 0 mM, 1.0 mM, 2.5 mM, 5.0 mM, and 10.0 mM ALA. Photoirradiation time was set at (A) 120 minutes, (B) 90 minutes, (C) 60 minutes, and (D) 30 minutes for accumulating light doses of 216 J/cm^2, 162 J/cm^2, 108 J/cm^2, and 54 J/cm^2, respectively. All data are expressed as the mean ± standard deviation.

120 minutes, respectively (Fig. 3). Nevertheless, cell proliferations took place once the photoirradiation was stopped, which means that PDI mediated by 1.0 mM ALA seemed to be ineffective against *P. aeruginosa*. While increasing the administration of ALA to 2.5 mM, 3.0 log, 4.1 log, 5.3 log, and 6.3 log of cells were killed when irradiated with 30 minutes, 60 minutes, 90 minutes, and 120 minutes of light, respectively. The cell proliferations could still be observed after the LED light source was removed. Treatment with 5 mM of ALA gave approximately 6.5-log reduction in the viable count if the light dose was 162 J/cm^2; no surviving cells could be detected if 10 mM of ALA was administered with the same light dose of 162 J/cm^2 (Fig. 3B). These data suggested that 5 mM of ALA with a 162 J/cm^2 light dose may be useful for PDI against *P. aeruginosa*.

3.4. *Survival fractions of S. aureus and P. aeruginosa*

The survival fractions were calculated by counting the number of CFUs to confirm the efficacy of ALA-medicated PDI again *S. aureus* and *P. aeruginosa*. As shown in Fig. 4, both survival fractions of *S. aureus* and *P. aeruginosa* were decreased with increasing ALA concentrations under the same light exposure (162 J/cm^2). The Gram-positive organism *S. aureus* appeared to be significantly more sensitive to 5-ALA-mediated PDI than the Gram-negative strains (e.g., survival fraction, −8.0 vs. −5.3 when administered 2.5 mM of ALA). By contrast, the survival fraction of *S. aureus* reached a plateau value (survival fraction: −8.0) when the lower concentration of ALA was administered (2.5 mM), whereas it needed 10 mM of ALA administered in order to reach the same efficiency of PDI when used against *P. aeruginosa*. To summarize, our results are in line with the statement from the literature as mentioned earlier. The main reason for the effects of ALA-mediated PDI on Gram-negative and Gram-positive bacteria is attributed to the complex outer membrane of Gram-negative bacteria acting as a barrier that hinders PS to transport through the cell membrane; thus, Gram-negative bacteria appear to be less sensitive to the lethal action of PDI with exogenously supplied porphyrins [7–9]. Furthermore, the results also correlated well with the previous data obtained from a microplate autoreader.

Although the addition of ALA with higher concentrations showed somewhat dark toxicities on *P. aeruginosa*, the efficacy

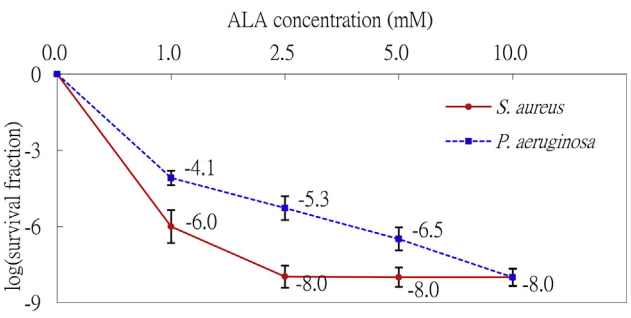

Fig. 4 – Log survival fractions of *Staphylococcus aureus* and *Pseudomonas aeruginosa* treated with 5-aminolevulinic acid (ALA) at various concentrations after 90 minutes of light exposure (162 J/cm^2).

of PDI mediated by ALA against *S. aureus* was still higher than that against *P. aeruginosa*. This phenomenon may be attributed to the fact that Gram-positive strains accumulate much more PAPs than Gram-negative strains: as more PAPs were accumulated, the more singlet oxygen was produced upon illumination and thus inactivated the bacterial cells [19].

The traditional enumeration of bacteria by direct plate counting on nutrient agar medium requires laborious dilution, an incubation process that is complicated and time consuming. Our study thus provides an efficient and reliable way for rapid screening the effects of PDI induced by ALA against microorganism species by measuring the apparent OD with a microplate autoreader.

4. Conclusions

The present study suggests that ALA in combination with red LED light is a potential candidate for PDI against *S. aureus* and *P. aeruginosa*. The use of OD to estimate CFUs in cell suspensions is proven to be a rapid, low-cost, and nondestructive alternative for optimizing the experimental conditions of ALA-induced PDI on bacterial strains. We believe that microplate-based assays could represent a very good alternative to the conventional colony count method in testing the antibacterial potential of different photocatalytic and other materials.

Conflicts of interest

All authors declare no conflicts of interest.

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