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ORIGINAL ARTICLE

Effects of toluidine blue O (TBO)-photodynamic inactivation on community-associated methicillin-resistant *Staphylococcus aureus* isolates

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Abstract *Background/objectives:* Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) was recognized as a leading pathogen and has been shown to be genetically different from the health care-associated MRSA (HA-MRSA). Photodynamic therapy (PDT) is considered a potential alternative method for the treatment of resistant bacterial infections, but the effect of PDT on CA-MRSA is unknown. The purpose of this study was to compare the bactericidal effects of toluidine blue O (TBO) on CA-MRSA and HA-MRSA and investigate the photodynamic inactivation effects of TBO (TBO-PDI) against bacterial virulence factors.
Materials and methods: TBO-PDI effects were determined by measuring the survival fractions for four strains and bactericidal activities for 26 CA-MRSA isolates and 26 HA-MRSA isolates. The

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influences of TBO-PDI on DNA fragmentation and the activities of protease, lipase, staphylococcal α-hemolysin, and enterotoxin were studied.
Results: TBO-PDI has effective bactericidal activity against both CA- and HA-MRSA. However, the bactericidal activity of TBO-PDI was significantly higher against HA-MRSA than CA-MRSA isolates. In addition, TBO-PDI treatment using a sublethal TBO concentration led to reduced production of several virulence factors, including protease, lipase, staphylococcal α-hemolysin, and enterotoxin.
Conclusion: Although TBO-PDI is slightly less effective against CA-MRSA than HA-MRSA isolates, TBO-PDI could reduce the production of virulence factors at a sublethal TBO concentration, which would be beneficial for treating CA-MRSA infections.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections are difficult to treat and are major clinical problems. During the past 10 years, the prevalence of hospital associated MRSA (HA-MRSA) in Taiwan has increased.[1] In addition, community-associated (CA)-MRSA was recognized and found to be genetically different from HA-MRSA.[2,3] HA-MRSA frequently infects elderly patients, commonly displays multidrug resistance, and carries a type I, II, or III SCC*mec* cassette. By contrast, CA-MRSA usually infects younger patients, and is more susceptible to non β-lactam antibiotics, and usually carries a type IV or V SCC*mec* cassette.[2,4,5]

CA-MRSA frequently causes skin and soft tissue infections (SSTIs)[6,7] and sometimes results in severe infections such as necrotizing pneumonia or sepsis.[2,8] A retrospective study showed that treatment failure for CA-MRSA SSTIs was mainly due to inappropriate MRSA therapy.[9] The authors reported that treatment failure occurred in 16 of 312 patients (5%) who received an active antibiotic compared to 29 of 219 patients (13%) who received an inactive antibiotic ($p = 0.001$; Chi-square analysis). However, active antibiotic therapy can only be started after the results of the antibiotic susceptibility test are available. This process takes time and is performed by either bacteria culture-based methods or through an automated system. Photodynamic inactivation (PDI) may be an alternative therapeutic option for the treatment of SSTIs because it has several benefits. First, it is a local treatment and thus does not provoke systemic effects in humans. Second, no PDI-resistant bacteria have been reported, and third, low-cost light sources are available.[10,11]

The mechanism of PDI was activation of nontoxic photosensitizer (toluidine blue O), which was exposed to appropriate wavelength, consequently providing reactive oxygen species including free radicals and singlet oxygen. These reactive oxygen species can damage DNA and bacterial cell membrane, leading to bacterial death.[10] PDI has been shown to effectively inactivate or kill microbial pathogens.[12–16] and has been used for chemical disinfection[17,18] or plaque-disclosing agents.[19] Various photosensitizers, such as hematoporphyrin,[20] phenothiazinium salts,[21] chlorin,[22,23] or 5-aminolevulinic acid,[21,24] have been used in PDI and has been shown to be bactericidal against *S. aureus*. Comparison of the bactericidal effects of PDI against MRSA and methicillin-susceptible *Staphylococcus aureus* (MSSA) has been previously studied,[25,26] but very few reports focused on CA-MRSA. In this study, we compared the bactericidal effects of TBO-PDI on CA-MRSA and HA-MRSA and investigated the efficacy of TBO-PDI against bacterial virulence factors such as protease, lipase, staphylococcal α-hemolysin, and enterotoxin type B.

Materials and methods

Bacterial strains

CA-MRSA isolates ($N = 26$) and HA-MRSA isolates ($N = 26$) were collected from the Bacteriology Laboratory in the National Taiwan University Hospital (NTUH, Taipei), a university hospital with 2,500 beds in northern Taiwan. CA-MRSA isolates ($N = 26$) were recovered from various specimens as described in our previous study[27] and HA-MRSA isolates were recovered from blood cultures.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using disc diffusion according to the guidelines from the Clinical and Laboratory Standards Institute (CLSI).[28] The following antimicrobial agents were tested: oxacillin (by cefoxitin), gentamicin, clindamycin, erythromycin, teicoplanin, minocycline, trimethoprim-sulfamethoxazole, and vancomycin.

*SCC*mec typing

The SCC*mec* types of the 26 CA-MRSA and 26 HA-MRSA clinical isolates were determined by types of the *ccr* genes and class of *mec* complexes using polymerase chain reaction and/or sequencing, which has been previously described.[29]

Killing curve of TBO-PDI on MRSA isolates

The PDI assay was performed as described in our previous study.[14] Briefly, bacteria were grown in 20 mL of trypticase soy broth (BD BBL, Franklin Lakes, NJ, USA) to an optical

density of approximately 0.6 at 600 nm. The bacteria were then harvested by centrifugation at 5,600 × *g* for 15 minutes and resuspended in phosphate buffered saline to a concentration of 10^8 colony forming unit (CFU)/mL. The bacterial suspensions were then incubated with a final concentration of TBO (0μM, 0.25μM, 0.5μM, 0.75μM, 1μM, and 2μM) and placed in 96-well microtiter plates. After incubation in the dark at room temperature (RT) for 20 minutes, the bacterial suspensions were exposed to 20 J of red light/cm^2 (630 nm, 50 mW/cm^2 for 6 minutes and 40 seconds) or kept in the dark. The light source consisted of a high power light-emitting diode (LED) array with the wavelength centered at 630 ± 5 nm and a bandwidth of 20 nm. The LED light source was fed by a small Direct Current (DC)-power supply. The size of the LED array is able to cover a 96-well plate with a power density of 50 mW/cm^2. The amount of bacteria that survived was assessed using the colony counting method. The survival fraction was defined as the ratio of bacterial CFU's posttreatment to pretreatment.

DNA fragmentation analysis

Bacterial cells (10^9 CFU/mL) were incubated with various TBO concentrations (0μM, 0.25μM, 0.5μM, 1μM, and 2μM) and placed in 96-well microtiter plates. After incubation in the dark at RT for 20 minutes, the bacterial suspensions were either exposed to 20 J of red light/cm^2 or kept in the dark. After treatment, the bacterial DNA was prepared and analyzed via pulsed-field gel electrophoresis (PFGE) as described previously.[14] The DNA was then separated on a CHEF-DRIII unit (Bio-Rad Laboratories, Hercules, CA, USA). Optimal separation was achieved using a pulse time of 5—30 seconds for 20 hours at 6 V/cm.

Protease and lipase activities assay

The protease and lipase activities were determined using the plate method.[15,30] Wells were dug into skim milk and Salt-Tween agar plates using a 6-mm, 200 μL pipette tip. The bacterial suspensions (10^8 CFU/mL) were incubated with varying TBO concentrations (0μM, 0.25μM, 0.5μM, 1μM, and 2μM) and placed in 96-well microtiter plates. After incubation in the dark at RT for 20 minutes, the bacterial suspensions were either exposed to 20 J of red light/cm^2 or kept in the dark. In order to make sure all tests containing same bacteria concentration, 40 μL of the bacterial suspensions (after incubation with various TBO concentrations) containing 10^5 CFU/mL were placed into each well. The plates were incubated at 37°C for 48 h. Then, the diameters of the hydrolyzed zone were measured. The experiments were performed in triplicate. The skim milk agar contained 5.0 g/L skim milk, 10.0 g/L yeast extract, and 12.5 g/L Bacto agar. The Salt-Tween agar contained 10.0 g/L peptone, 10.0 g/L yeast extract, 75.0 g/L NaCl, 0.1 g/L $CaCl_2H_2O$, 10.0 g/L Tween 80, and 15.0 g/L Bacto agar, pH 7.2.

Western blot analysis of staphylococcal enterotoxin type B and α-hemolysin

After treatment with PDI as previously described, the bacteria were disrupted using sonication and centrifuged at 15,000 × *g*. The supernatant was collected as the protein sample and stored at −20°C until use. The protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories) with Bovine Serum Albumin (BSA) as the standard. Samples containing equal amounts of cellular proteins were separated on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel, and the gels were then electroblotted into Immobilon-NC transfer membranes (Millipore, Bedford, MA, USA). Enterotoxin type B and α-hemolysin were detected by Western blot analysis using a 1:10000 dilution of rabbit anti-Staphylococcal enterotoxin B (SEB) affinity purified immunoglobulin G (IgG) and sheep anti-α-hemolysin affinity purified IgG, which were purchased from Toxin Technology (Sarasota, FL, USA). The blots were then washed and incubated with a 1:10000 dilution of goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugated secondary antibody or rabbit anti-sheep IgG HRP conjugated secondary antibody (Sigma Aldrich, St. Louis, MO, USA). The proteins were detected using the enhanced chemiluminescence (ECL) detection system (ECL Western Blotting Substrate, Pierce Biotechnology, Rockford, IL, USA). Bands were scanned and quantified with the gel analysis software Image (Quantity One; Bio-Rad Laboratories).

Bactericidal activities on CA-MRSA and HA-MRSA isolates

The bactericidal activity assay was slightly modified from our previous study.[14] Briefly, the overnight bacterial suspensions were adjusted to 10^8 CFU/mL, added to 96-well microtiter plates, and incubated with TBO at 1μM. After incubation in the dark at RT for 20 minutes, the bacterial suspensions were exposed to 20 J of red light/cm^2 or kept in the dark. Subsequently, 20 μL of the bacterial suspension from each well was placed onto Mueller—Hinton agar (MHA; Difco, Detroit, MI, USA) for 20 hours at 37°C.

Statistical analysis

The GraphPad Prism software (GraphPad Prism Software Inc., San Diego, CA, USA) was used for the statistical analysis. One-way analysis of variance (ANOVA) with correction for the multiple comparisons (Bonferroni *post hoc* test) was used to test the difference between killing curves of TBO-PDI on five MRSA strains. A nonparametric test (Mann—Whitney *U* test) was used to test the difference between effect of TBO-PDI on plasmid degradation, protease activities, lipase activities, staphylococcal α-hemolysin and enterotoxin type B. A p value < 0.05 was considered significant.

Results

Characterization of the isolates

For 26 CA-MRSA isolates, 11 harbored type IV SCC*mec* elements and 11 harbored type V* SCC*mec* elements. Most of the (21/26) HA-MRSA isolates harbored type II ($n = 10$) or III ($n = 11$) SCC*mec* types. The gentamicin resistance rates

Table 1 Characteristics of community-associated and hospital-associated methicillin-resistant *Staphylococcus aureus* clinical isolates

MRSA	Specimen	SCC*mec*	Antibiotic resistance (No. of isolates)				
			CC	E	GM	SXT	MI
Community-associated (n = 26)	Abscess (1); bronchial washing (1); blood (1); discharge (2); ear (1); eye (2); pus (5); skin pus (3); sputum (5); surgical wound (2); throat swab (1); urine (2)	II (1); III (3); IV (11); V*(11)	22	22	13	4	3
Hospital-associated (n = 26)	Blood (26)	II (10); III (11); IV (2); V*(3)	24	24	22	11	7

All were resistant to oxacillin, but susceptible to teicoplanin and vancomycin.
CC = clindamycin; E = erythromycin; GM = gentamicin; MI = minocycline; MRSA = methicillin-resistant *Staphylococcus aureus*; SXT = trimethoprim-sulfamethoxazole.

were different between the CA-MRSA (13/26) and HA-MRSA (22/26) groups (Table 1).

Killing curves of TBO-PDI on five MRSA strains

The killing curves of TBO-PDI were determined on five MRSA strains (ATCC 33592, 1 HA-MRSA, and 3 CA-MRSA isolates). ATCC 33592 and HA-MRSA_MY8703 carry the SCC*mec* III element, and CA-MRSA _194 carry SCC*mec* IV, CA-MRSA_3, and CA-MRSA R32 contain SCC*mec* V* element, respectively. TBO at a concentration of $\leq$ 2μM without light did not have bactericidal activity (Fig. 1). The bactericidal activity of TBO-PDI was examined by incubating the bacteria with different concentrations of TBO (0μM, 0.25μM, 0.5μM, 0.75μM, 1μM, and 2μM) for 20 minutes at RT and then exposing them to 20 J of red light illumination (Fig. 1). A strong bactericidal effect that resulted in a 6—7-log reduction of bacterial cells was found at a TBO concentration of 2μM for all five strains. Slightly different bactericidal activities were found among isolates (ATCC 33592 ≒ HA-MRSA_MY8703 > CA-MRSA_3 ≒ CA-MRSA_R32 > CA-MRSA _194) when the TBO concentrations were 0.5μM, 0.75μM, and 1μM (Fig. 1). At a TBO concentration of 0.5μM, CA-MRSA_3, CA-MRSA_R32, and

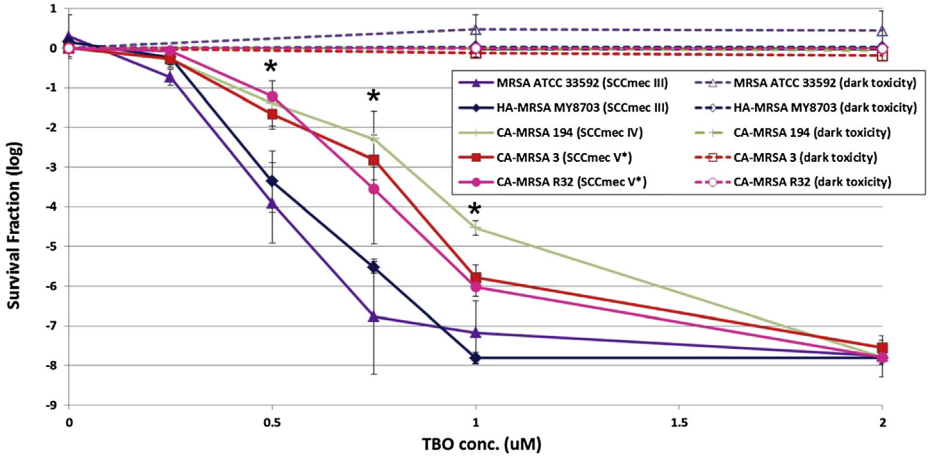

Figure 1. Survival fractions of MRSA bacteria (10^8 cells/mL) incubated with various concentrations of toluidine blue O (TBO) followed by dark or by light illumination: CA-MRSA _194, CA-MRSA_3, CA-MRSA_R32, HA-MRSA_MY8703, and ATCC 33592 were incubated with various concentrations of TBO for 20 minutes at RT and were then kept in the dark or exposed to 20 J of red light/cm^2. Each data point represents the mean ± standard error of three experiments. The statistical analysis was performed using a one-way analysis of variance (GraphPad Prism software) to correct for multiple comparisons (Bonferroni multiple comparison test; *, $p < 0.05$). Filled square = CA-MRSA_3 with varying concentrations of TBO followed by illumination, empty square = CA-MRSA_3 with varying concentrations of TBO that was kept in the dark, filled circle = CA-MRSA_R32 with varying concentrations of TBO followed by illumination, empty circle = CA-MRSA_R32 with varying concentrations of TBO that was kept in the dark, filled cross = CA-MRSA_194 with varying concentrations of TBO followed by illumination, empty cross = CA-MRSA_194 with varying concentrations of TBO that was kept in the dark, filled diamond = HA-MRSA MY8703 with varying concentrations of TBO followed by illumination, empty diamond = HA-MRSA_MY8703 with varying concentrations of TBO that was kept in dark, filled triangle = MRSA ATCC33592 with varying concentrations of TBO followed by illumination, empty triangle = MRSA ATCC33592 with varying concentrations of TBO that was kept in the dark. CA-MRSA = community-associated methicillin-resistant *Staphylococcus aureus*; HA-MRSA = healthcare-associated methicillin-resistant *Staphylococcus aureus*.

CA-MRSA _194 showed only 1.7-log, 1.2-log, and 1.4-log reductions of viability, but ATCC33592 and HA-MRSA_MY8703 showed 3.9-log and 3.3-log reductions in viability, respectively. At a TBO concentration of 0.75μM, CA-MRSA_3, CA-MRSA_R32, and CA-MRSA _194 showed 2.8-log, 3.5-log reductions and 2.3-log of viability, but ATCC33592 and HA-MRSA_MY8703 showed 6.8-log and 5.5-log reductions in viability, respectively. At a TBO concentration of 1μM, CA-MRSA_3, CA-MRSA_R32, and CA-MRSA _194 showed 5.8-log, 6.0-log, and 4.5-log reductions and ATCC33592 and HA-MRSA_MY8703 showed 7.2-log and 7.8-log reductions in viability, respectively. Thus, TBO-PDI had a greater bactericidal effect on HA-MRSA_MY8703 and ATCC33592 than on CA-MRSA isolates. These differences were statistically significant as determined by a one-way ANOVA (GraphPad Prism software) with correction for the multiple comparisons (Bonferroni *post hoc* test).

Effect of TBO-PDI on DNA fragmentation

TBO-PDI treatment resulted in chromosomal DNA fragmentation (50—100 kb) and plasmid degradation in MRSA. In particular, plasmid degradation was dependent on the TBO dose (Fig. 2). Plasmid degradation of MRSA were reduced after PDI with TBO concentrations of 0.5μM, 1μM, and 2μM ($p < 0.05$ using the Mann—Whitney *U* test). The intensity of the compression zone (arrow in Fig. 2) increased with greater TBO concentrations. These results are consistent with our previous finding in *Pseudomonas aeruginosa*, in that the bacterial chromosomal DNA and plasmids were photo-damaged by TBO-PDI.[14]

Effect of TBO-PDI on protease and lipase activities

CA-MRSA_3, isolated from skin pus, represents a major clone of CA-MRSA isolates analyzed by PFGE.[27] Thus we used this strain for the testing. Production of virulence factors of CA-MRSA_3 were tested by TBO-PDI treatment under the sub-lethal TBO concentration. After TBO-PDI treatment, the bacterial suspensions were adjusted from 10^8 CFU/mL to 10^5 CFU/mL and 40 μL of the bacterial suspensions were placed into each well. The hydrolyzed zones were measured to determine the protease and lipase activities. Negative control, dark control, and light control conditions had similarly sized hydrolyzed zones for the protease and lipase activities (Fig. 3). The protease activity after TBO-PDI was significantly decreased with a TBO concentration of 0.25 μM ($p < 0.05$ by Mann—Whitney *U* test), whereas the lipase activity was slightly decreased at a TBO concentration of 0.25 μM. Both the protease and lipase activities of MRSA were reduced after PDI with TBO concentrations of 0.5μM, 1μM, and 2μM ($p < 0.05$ using the Mann—Whitney *U* test).

Effect of TBO-PDI on staphylococcal α-hemolysin and enterotoxin type B

The effects of TBO-PDI on the staphylococcal α-hemolysin and enterotoxin type B were determined by Western blot analysis (Figs. 4 and 5). Similar levels of α-hemolysin and

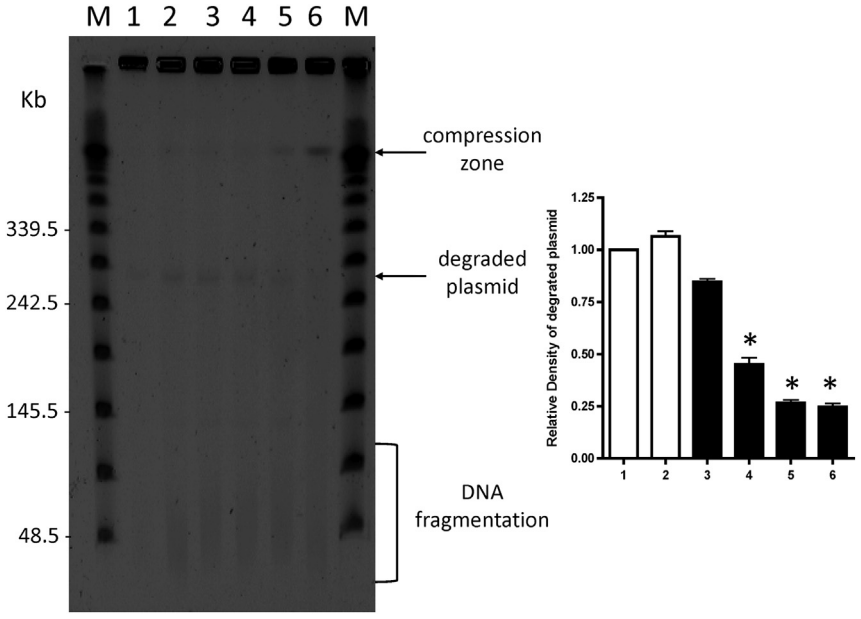

Figure 2. Chromosomal DNA fragments were analyzed by pulsed-field gel electrophoresis: CA-MRSA 3 (10^9 CFU/mL) bacteria were treated with toluidine blue O (TBO) for 20 minutes at room temperature (RT) with or without illumination of 20 J of red light/cm^2. Lane M, DNA size marker (lambda ladder PFG marker); Lane 1, LC, light control (630 nm, 20 J of red light/cm^2, no TBO); Lane 2, DC, dark control (2μM of TBO, no light); Lane 3, photodynamic inactivation (PDI) with TBO (0.25μM) and light (20 J); Lane 4, PDI with TBO (0.5μM) and light (20 J); Lane 5, PDI with TBO (1μM) and light (20 J); Lane 6, PDI with TBO (2μM) and light (20 J). The arrow represents the compression zone and the degraded plasmid. Each bar represents the mean ± standard error of three experiments. Data are expressed relative to the DC values, which are arbitrarily set at 1.0. * $p < 0.05$ versus dark control group (statistical analysis using Mann—Whitney *U* test). CA-MRSA = community-associated methicillin-resistant *Staphylococcus aureus;* HA-MRSA = healthcare-associated methicillin-resistant *Staphylococcus aureus*.

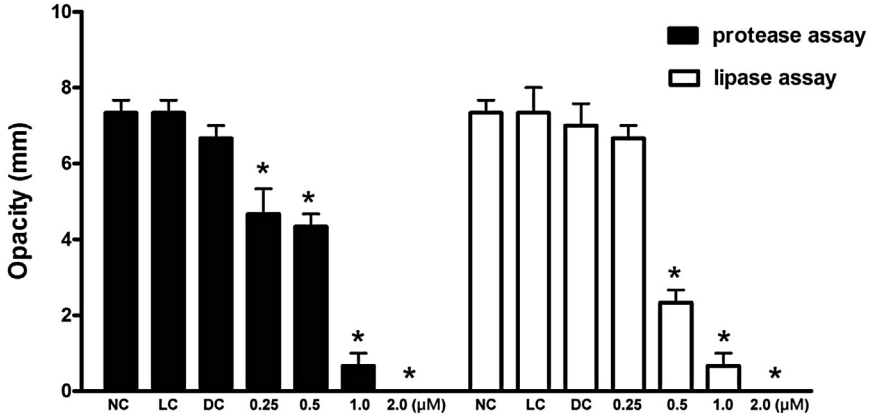

Figure 3. The protease and lipase activities were determined using the plating method: community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) 3 (10^8 CFU/mL) bacteria were treated with various concentrations of toluidine blue O (TBO) for 20 minutes at room temperature (RT) with or without illumination of 20 J of red light/cm^2. Bacterial suspensions were adjusted to 10^5 CFU/mL and 40 μL of the bacterial suspensions were placed into each well. Photodynamic inactivation (PDI) with different concentrations of TBO and light (20 J) were shown in different bars. Each bar represents the mean ± standard error of three experiments. Asterisks indicate a statistically significant decrease ($p < 0.05$ using the Mann–Whitney *U* test). DC = dark control (2μM of TBO, no light); LC = light control (630 nm, 20 J of red light/cm^2, no TBO); NC = native control (without TBO and illumination).

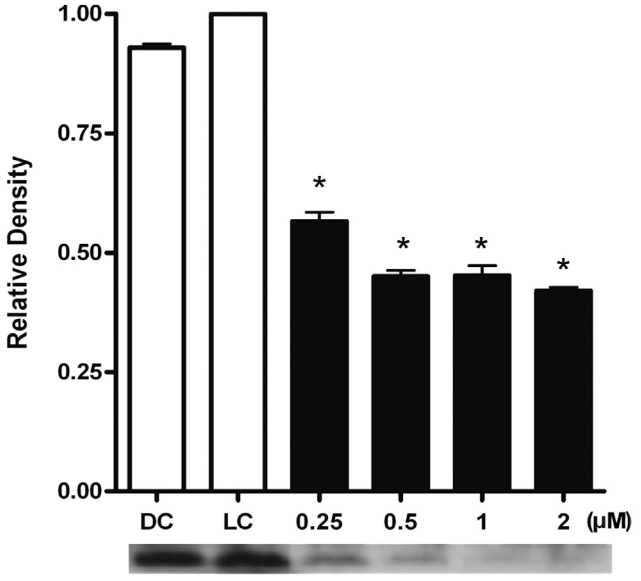

Figure 4. Photodynamic inactivation (PDI) effects on staphylococcal α-hemolysin were measured by Western blot analysis: community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) 3 (10^8 CFU/mL) were treated with 0μM, 0.25μM, 0.5μM, 1μM, and 2μM of toluidine blue O (TBO) for 20 minutes at room temperature (R) with or without illumination of 20 J of red light/cm^2. PDI with TBO (0.25μM, 0.5μM, 1μM, and 2μM of TBO) and light (20 J) were shown in different bars. Each bar represents the mean ± standard error of three experiments. Data are expressed relative to the LC values, which are arbitrarily set at 1.0. * $p < 0.05$ versus LC group (statistical analysis using Mann–Whitney *U* test). DC = dark control (4μM of TBO, no light); LC = light control (630 nm, 20 J of red light/cm^2, no TBO).

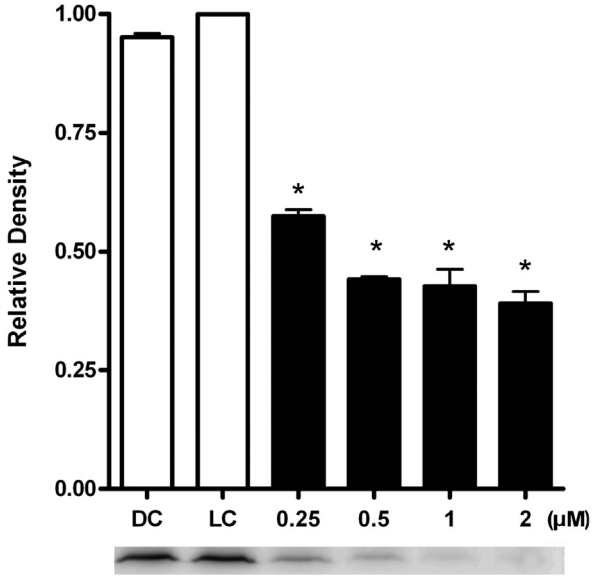

Figure 5. Photodynamic inactivation (PDI) effects on staphylococcal enterotoxin b were measured by Western blot analysis: community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) 3 (10^8 CFU/mL) were treated with 0μM, 0.25μM, 0.5μM, 1μM, and 2μM of toluidine blue O (TBO) for 20 minutes at room temperature (RT) with or without illumination of 20 J of red light/cm^2. PDI with TBO (0.25μM, 0.5μM, 1μM, and 2μM of TBO) and light (20 J) were shown in different bars. Each bar represents the mean ± standard error of three experiments. Data are expressed relative to the LC values, which are arbitrarily set at 1.0. * $p < 0.05$ versus LC group (statistical analysis using Mann–Whitney *U* test). DC = dark control (4μM of TBO, no light); LC = light control (630 nm, 20 J of red light/cm^2, no TBO).

enterotoxin type B activities were observed in the dark control or light control conditions (Figs. 4 and 5). After TBO-PDI treatment, the tested CA-MRSA strain showed a significant reduction in the expression of α-hemolysin and enterotoxin type B ($p < 0.05$ using the Mann–Whitney *U* test). It should be noted that only a 0.28-log reduction of bacterial cells counts was observed with a TBO concentration of 0.25μM (Fig. 1). These data indicate that TBO-PDI inhibited the expression of virulence factors at sublethal TBO concentrations.

Comparison of bactericidal activity of TBO-PDI against CA-MRSA and HA-MRSA isolates

Because of the genetic differences between the CA-MRSA and HA-MRSA isolates, the efficacy of TBO-PDI against the strains may not be the same. Thus, we tested bactericidal activity on CA-MRSA and HA-MRSA isolates. Our results indicate the majority of HA-MRSA responded better to TBO-PDI than CA-MRSA (Table 2). Comparison of bactericidal activity on different SCC*mec* types of isolates indicates that SCC*mec* type IV and V* (22 CA-MRSA and 5 HA-MRSA) showed less sensitive than SCC*mec* II and III (4 CA-MRSA and 21 HA-MRSA) (Table 2).

Discussion

Increasing resistance to antibiotics in MRSA is a global concern. For this reason, PDI, for which the bactericidal effect works through a different mechanism, has been considered an alternative and effective therapeutic option.[17,18] Previous reports have shown bactericidal activity of PDI with TBO or methylene blue (MB) against MRSA isolates.[12,26] However, most studies focused on comparing the effects of PDI between MRSA and MSSA strains, whereas the efficacy of PDI against CA-MRSA was largely unknown. Thus, in the current study, we focused on comparing PDI against CA-MRSA and HA-MRSA isolates. For more clinical application, we used epidemiological criteria for defining the HA- and CA-MRSA. The emergence of MRSA with CA genotypes (i.e., SCC*mec* IV, V) strains as nosocomial isolates may complicate the compositions of CA- and HA-MRSA strains. Our results indicate that TBO-PDI significantly but differentially inhibited the survival of both CA-MRSA and HA-MRSA. By killing curve assay of two HA-MRSA and three CA-MRSA, as well as bactericidal activities against more isolates (a total of 26 CA-MRSA and 26 HA-MRSA) support this finding. If compared by SCC*mec* types, isolates harboring SCC*mec* II or III (HA-MRSA) were slightly more sensitive to PDI than strains harboring SCC*mec* IV or V (CA-MRSA). One study found that PDI activity against *S. aureus* was correlated with the specific *agr*/SCC*mec* pattern.[31] The other study illustrated that clonal complex 1 accounted for elevated resistance and CC30 (ST36) for susceptibility to photoinactivation.[32] The reason for different activity against CA-MRSA and HA-MRSA is unknown, but may be due to different genetic backgrounds including the sequencing types and SCC*mec* types.[2,3] Another possibility is the difference of specimen (HA-MRSA isolates were from blood and CA-MRSA isolates were from various specimens), and this difference could also be related to different genetic background of isolates.

Photodamage to the DNA and the cytoplasmic membrane have been proposed as possible mechanisms of PDI in bacteria.[10] An *in vitro* study using hematoporphyrin-PDI against *S. aureus* demonstrated that PDT resulted in photodamage to cytoplasmic membrane proteins, chromosomal and plasmid DNA.[20] Furthermore, PDI may also induce oxidative stress-derived DNA damage and lead to the production of 7,8-dihydro-8-oxo-20-deoxyguanosine (8-oxodG).[33] This modification of guanidine could cause DNA misreplication and fragmentation.[34] Our study showed that PDI treatment caused chromosomal DNA fragmentation (50–100 kb) and plasmid DNA degradation (Fig. 2).

To reduce the selective pressure for the development of drug-resistant bacteria, antivirulence strategies and several compounds have been developed.[35] A similar strategy can be achieved using TBO-PDI. It has been reported that virulence factors are significantly reduced by TBO-PDI treatment in *Escherichia coli*, *P. aeruginosa*,[14,36] and *Porphyromonas gingivalis*[13]; however, the study of effect on staphylococcal virulence factors was limited in the literature. One study showed that PDI with phenothiazinum MB inhibited the purified protein activities of V8 protease, α- hemolysin, and sphingomyelinase in *S. aureus* in a dose-dependent manner.[37] In the current study, we found that TBO-PDI at a sub-lethal TBO concentration, as low as 0.25μM, inhibited the activities of the protease, lipase, staphylococcal α-hemolysin, and enterotoxin type B produced by CA-MRSA stains. These results indicate that even CA-MRSA_3 showed only a 1.7-log reduction of viability at a TBO concentration of 0.5μM, TBO-PDI still effectively reduced the production of virulence factors. A study by Tubby et al[37] also reported that extensive virulence factors could be reduced by photodynamic therapy. These results imply that it may be possible to treat CA-MRSA infections by using low-dose photosensitizer through inhibition of the production of virulence factors; even the bactericidal effect is only partial.[38]

Table 2 Comparison of bactericidal activities of toluidine blue O-photodynamic inactivation for 26 community-associated methicillin-resistant *Staphylococcus aureus* and 26 healthcare-associated methicillin-resistant *Staphylococcus aureus* clinical isolates

$\log_{10}$- unit reduction	Community-associated MRSA ($n = 26$)				Hospital-associated MRSA ($n = 26$)			
	SCC*mec* II	SCC*mec* III	SCC*mec* IV	SCC*mec* V*	SCC*mec* II	SCC*mec* III	SCC*mec* IV	SCC*mec* V*
< 3	0	2	11	11	0	6	2	3
≥ 3 (3 to 8)	1	1	0	0	10	5	0	0

MRSA = methicillin-resistant *Staphylococcus aureus*.

PDI has been previously shown to effectively reduce bacteria in surgical and burn wound models that were infected with an HA-MRSA strain.[39,40] Similar animal models may be used to test the effectiveness on CA-MRSA infections. If we can successfully treat these infections, we may decrease the incidence of serious CA-MRSA infections and reduce health care costs. In addition, the strength of reducing bacterial virulence factors under low dose of PDI indicates the effective strategy for treating SSTIs caused by CA-MRSA.

Conflicts of interest

All authors report no conflicts of interest relevant to this article.

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