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Photodynamic inactivation of multi-drug resistant pathogens in Hong Kong

Key Messages

1. Photodynamic therapy could be an alternative treatment for highly prevalent local antibiotic-resistant pathogens.
2. Photodynamic inactivation using toluidine blue O was observed on both reference strains and clinical samples, including methicillin-resistant *Staphylococcus aureus*, extended-spectrum β-lactamase–producing *Escherichia coli* and *Klebsiella pneumoniae*, *Candida glabrata* and *Candida krusei*.

Introduction

The widespread systemic use of antibiotics is a cause of multi-drug resistance and super-infection. In addition to multi-drug resistant (MDR) bacteria, fungal species such as *Candida glabrata* and *Candida krusei* have become increasingly prevalent due to resistance to traditional antifungal agents in immunocompromised patients.[1] Innovative methods to reduce MDR bacteria and fungi are required. Photodynamic inactivation (PDI) may be a useful approach in treating MDR bacteria and fungi.[2] It employs a non-toxic dye—photosensitiser (PS)—that selectively targets its destination cells while sparing the host cells. Upon illumination by specific wavelengths of visible light and in the presence of oxygen, reactive species are generated that destroy the pathogens. The extent of killing depends on the type of photosensitiser used, light dose, drug dose and the bacterial growth phase. There is also a difference in susceptability to PDI between Gram-positive and Gram-negative bacteria due to differences in physiology and cytoplasmic membrane protein structure.[2,3]

The efficiency of PDI depends on the match between PS and light illumination. The selectivity of PS to various bacteria is one of the key concerns of PDI. In general, neutral or anionic PS molecules bind efficiently to and photo-inactivate Gram-positive bacteria. In contrast, they may bind to the outer membrane only of Gram-negative bacteria, which are not inactivated. However, PSs with an overall cationic charge efficiently kill both Gram-positive and Gram-negative species. This has been shown for cationic porphyrins, phthalocyanines and phenothiazines.[4] Cationic phenothiazines such as methylene blue (MB) and toluidine blue O (TBO) have also been studied to photo-inactivate both Gram-positive and Gram-negative bacteria *in vitro* and in *ex vivo* samples.[2,5]

This study aims to elucidate the *in vitro* PDI efficacy of three PSs: MB, TBO, and delta-aminolevulinic acid (ALA) against highly prevalent antibiotic-resistant pathogens, namely methicillin-resistant *Staphylococcus aureus* (MRSA), extended-spectrum β-lactamase (ESBL) producing strains of *Escherichia coli* and *Klebsiella pneumoniae*, MDR *Pseudomonas aeroginosa*, *C krusei*, and *C glabrata*. One clinical isolate of each strain plus wild-type reference strains were comparatively studied.

The objectives of this study were: (1) to compare the *in vitro* PDI efficacy of the three PSs at different concentrations and light dose combination to these clinical MDR isolates, (2) to determine the minimum bactericidal concentration (MBC) of the three PSs on different pathogens, (3) to quantitate and compare the uptake kinetics of ALA-induced porphyrin for the proposed pathogens by flow cytometry (drug uptake is the major dependent factor for effective killing), and (4) to evaluate the PDI effect for the virulence factor, lipopolysaccharide (LPS), at pre- and post-PDI.

Methods

This study was conducted from February 2006 to December 2006. A total of eight bacterial and four fungal isolates were investigated. They included ATCC wild-type strains, clinical wild-type isolates, and clinical MDR isolates. Wild type strains included *S aureus* (ATCC 25923), *E coli* (ATCC 23922), *K pneumonia* (clinical wild type isolate), *P aeruginosa* (ATCC 27853), *C glabrata*

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(ATCC 90030) and *C krusei* (ATCC 6258). Clinical MDR isolates included MRSA, ESBL-producing Gram-negative pathogens included *E coli* and *K pneumonia*, *P aeruginosa*, *C glabrata* and *C krusei*. All clinical isolates were archived samples collected from the Department of Microbiology, United Christian Hospital, Hong Kong SAR, China.

Photosensitisers

Of the three PSs, MB is cationic, TBO is a phenothiazinium salt, which is also moderately effective cationic, and ALA is a pro-drug, which could increase the intra-cellular level of PpIX. Stock solutions of each PS were prepared by dissolving them in sterile distilled water, and then stored at 4ºC in the dark.

Study design

Regarding PDI efficacy of each PS on wild-type and MDR pathogens by MBC, bacterial strains (*S aureus*, *E coli*, *K pneumonia* and *P aeruginosa*) were grown in suspensions of cell density of $1x10^8$ cells/mL at 37ºC in nutrient broth, and *C glabrata* and *C krusei* isolates were grown in suspensions of a cell density of $1x10^6$ cells/mL at 37ºC in Sabouraud dextrose broth. Aliquots of bacterial suspensions were sensitised with a range of PS concentrations for 30 minutes. The PS-loaded cells were centrifuge-washed with sterile phosphate-buffered saline and resuspended in broth. Aliquots of 150 μL treated cells were placed in a 96-well microtitre plate and irradiated with light range (5-30 Jcm^{-2}) emitted from a 400-W quartz-halogen lamp equipped with a heat isolation filter and a long-pass filter (600 nm). Then 100 μL broth was withdrawn and serially diluted in phosphate-buffered saline; 10 μL from each dilution mixture was spread on nutrient agar plates in triplicate. The plates were incubated at 37ºC overnight. The number of colonies was enumerated to determine the survival fractions. Six independent experiments were conducted and the results were presented as mean and standard error of the mean (SEM) values. Light-alone controls (with no-PS) and dark controls (PS-treated cell suspension without light) were included.

Regarding bacterial uptake of ALA, all pathogens were sensitised with different drug concentrations and incubated for 4 hours for drug uptake. The fluorescence intensity of PpIX was determined by flow cytometry.

Regarding the PDI effect for the virulence factor of LPS using the limulus amoebocyte lysate (LAL) assay, the amount of LPS after MB, TBO and ALA mediated PDI was determined by the LAL assay (Associates of Cape Cod Inc, USA). In the presence of LPS, enzymes in the LAL assay can be activated and result in clot formation. Different concentrations of MB, TBO or ALA were separately mixed with LPS to attain a final LPS concentration of 0.25 or 0.03 EU/mL (LPS detection limits of LAL assay kits). After light inactivation, individual PS-LPS mixtures were incubated with the LAL. During the incubation, enzyme activation in the LAL was catalysed by the LPS resulting in clot formation. If more LPS is bound or destroyed by PDI, less free LPS remains to trigger the enzyme activation, resulting in no clot formation and vice versa. Positive controls were included with LPS alone at the concentration of 0.25 and 0.03 EU/mL. LAL reagent water from the commercial LAL kit served as a negative control. The experiments were conducted in triplicate.

Data analysis

Results were presented as mean and SEM values of six independent experiments. Statistical analyses were conducted by one-way ANOVA; a P value of <0.05 was considered statistically significant.

Results

Regarding the PDI efficacy of MB (Fig. a), with 3 μM MB at 30 Jcm^{-2}, 6.5 log killing was obtained for the *S aureus* (ATCC 25923) and 7 log killing was obtained for MRSA. With 8 μM MB at 30 Jcm^{-2}, 6 log killing was obtained for both clinical wild-type *E coli* and ESBL-producing *E coli*. With 10 μM MB at 30 Jcm^{-2}, 5.9 log killing was obtained for clinical wild-type *K pneumonia* and 4.8 log killing was obtained for ESBL-producing *K pneumonia*. However, MB-PDI demonstrated only 2.8 log killing for *P aeruginosa* MDR isolate at 200 μM MB at 30 Jcm^{-2}. Applying 1 μM MB at 30 Jcm^{-2}, no PDI effects were demonstrated for both MDR *Candida* species.

Regarding PDI efficacy of TBO (Fig. b), with 0.5 to 10 μM TBO and light doses (10, 20 and 30 Jcm^{-2}) applied to four groups of bacteria and two groups of fungi, a significant reduction ($P<0.01$) of viability count was noted. With 1 μM TBO at 30 Jcm^{-2}, 5.9 log killing was obtained for the *S aureus* (ATCC 25923) and 4.3 log killing for MRSA. With 2 μM TBO at 30 Jcm^{-2}, 6 log killing were obtained for both *E coli* (clinical wild-type) and ESBL-producing Gram-negative *E coli*. With 5 μM TBO at 30 Jcm^{-2}, 4.8 log killing was obtained for *K pneumonia* (clinical wild-type isolate) and 6 log killing for ESBL-producing *K pneumonia*. With 10 μM TBO at 30 Jcm^{-2}, 2.5 log killing was obtained for *P aeruginosa* (ATCC 27853) and 3.9 log killing for *P aeruginosa* clinical MDR isolate. With 100 μM TBO at 30 Jcm^{-2}, the *C glabrata* MDR isolates responded better with 4.6 log killing, compared to just 4 log killing for *C glabrata* (ATCC 90030). In contrast, only with 40 μM TBO at 15 Jcm^{-2}, near 5 log killing was obtained for both clinical MDR isolates and *C krusei* (ATCC 6258). These findings demonstrated that TBO mediated PDI was effective for both wild type and clinical MDR bacterial and fungal isolates. The poorest response was observed for *P aeruginosa* strains.

Regarding PDI efficacy of ALA (Fig. c), by applying up to 6 μM ALA at 30 Jcm^{-2}, all groups of pathogens (*S aureus*, MRSA, *E coli*, ESBL *E coli*, *K pneumonia*, MDR *K pneumonia*, *P aeruginosa* and MDR *P aeruginosa*) showed <1 log killing. This may be due to poor ALA uptake by

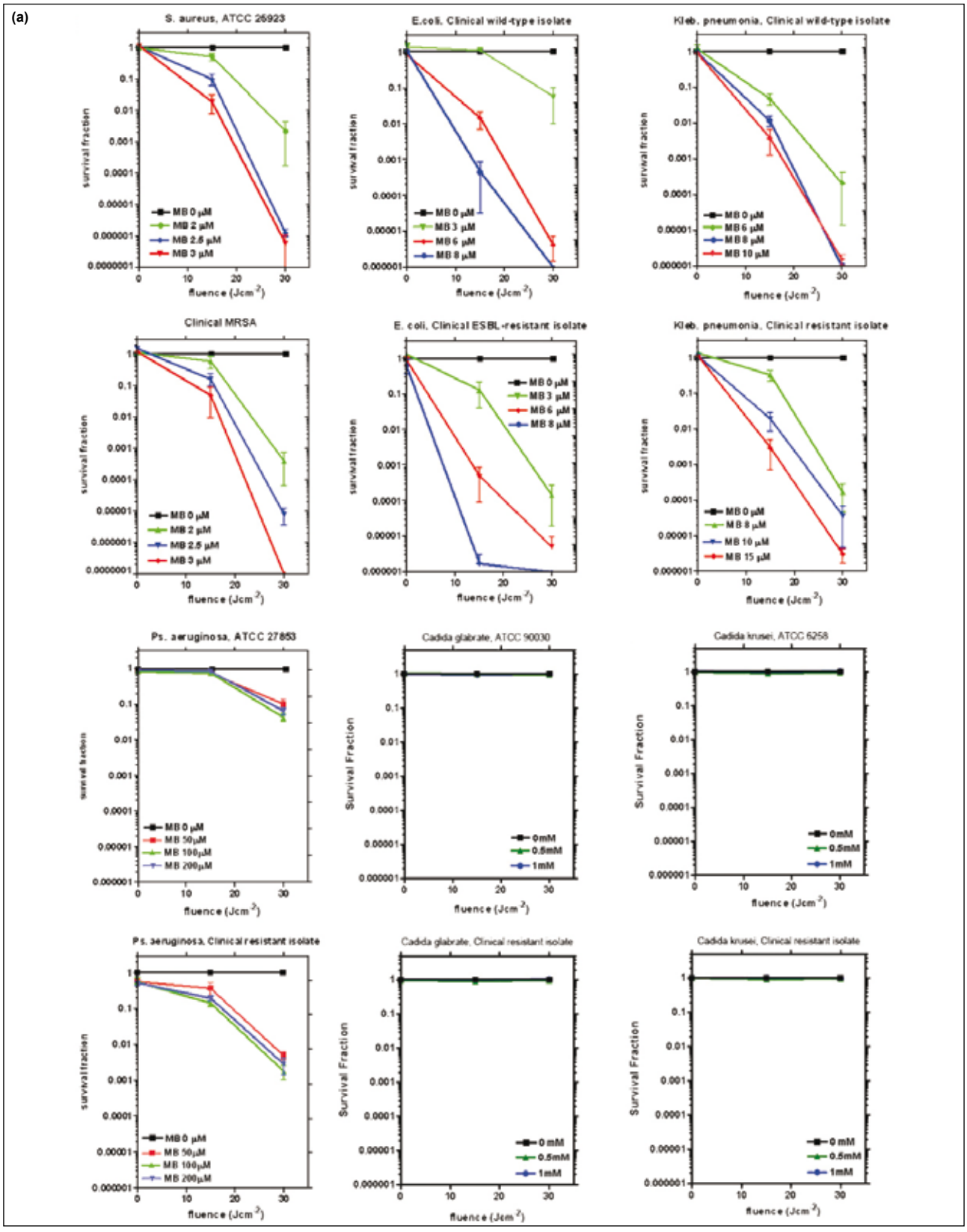
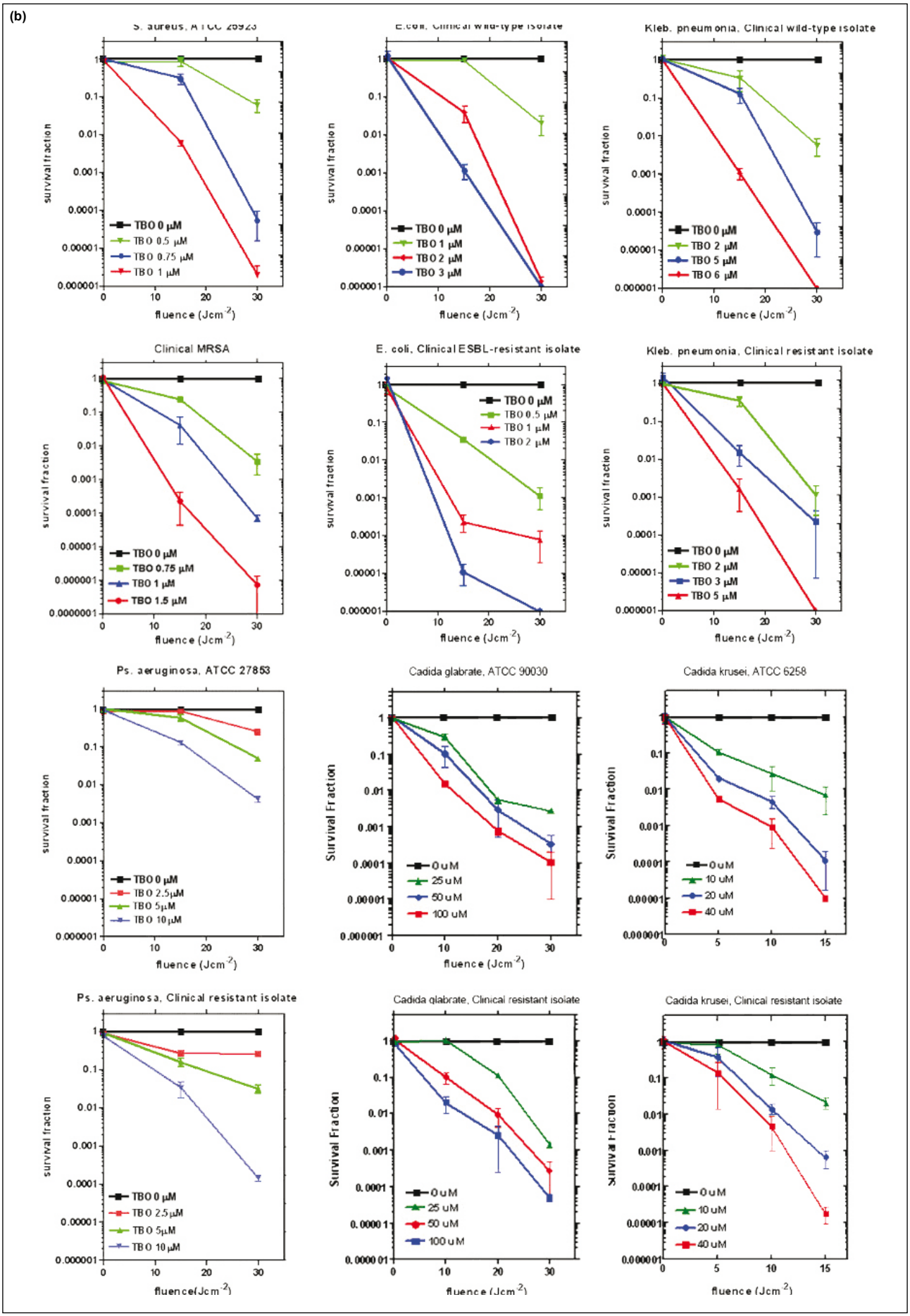
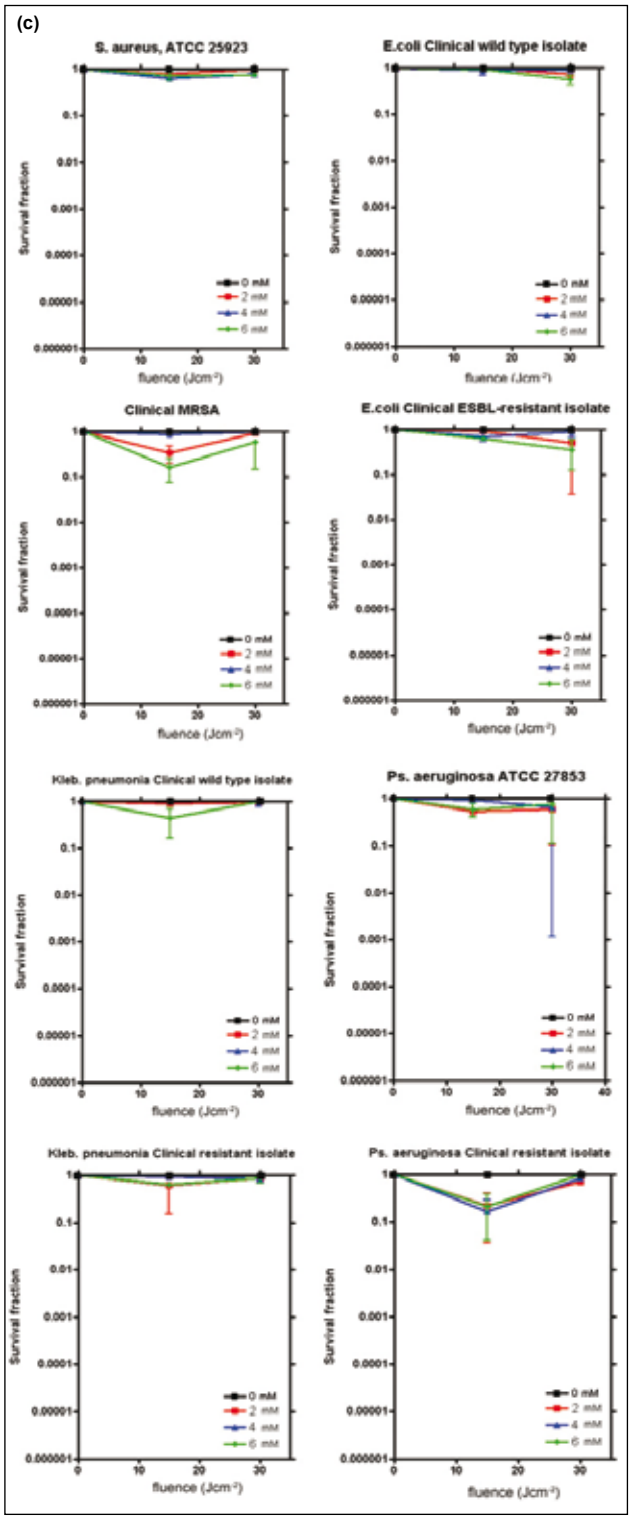


Fig. The photodynamic inactivation efficacy of (a) methylene blue, (b) toluidine blue O, and (c) delta-aminolevulinic acid against different pathogens using minimum bactericidal concentration

(1) *Staphylococcus aureus* (ATCC 25923) and methicillin-resistant *S aureus*, (2) *Escherichia coli* (clinical wild-type isolate) and extended-spectrum β-lactamase (ESBL)-producing Gram-negative *E coli*, (3) *Klebsiella pneumonia* (clinical wild-type isolate) and ESBL-producing Gram-negative *K pneumonia*, (4) *Pseudomonas aeruginosa* (ATCC 27853) and its clinical multidrug resistant (MDR) isolate, (5) *Candida glabrata* (ATCC 90030) and its clinical MDR isolate, and (6) *Candida krusei* (ATCC 6258) and its clinical MDR isolate. Results are presented as means and standard error of the means of six independent experiments (P<0.01).


(b)
S. aureus, ATCC 29923
survival fraction
fluence (Jcm-2)
TBO 0 μM
TBO 0.5 μM
TBO 0.75 μM
TBO 1 μM
E.coli, Clinical wild-type isolate
TBO 0 μM
TBO 1 μM
TBO 2 μM
TBO 3 μM
Kleb. pneumonia, Clinical wild-type isolate
TBO 0 μM
TBO 2 μM
TBO 5 μM
TBO 6 μM
Clinical MRSA
TBO 0 μM
TBO 0.75 μM
TBO 1 μM
TBO 1.5 μM
E. coli, Clinical ESBL-resistant isolate
TBO 0 μM
TBO 0.5 μM
TBO 1 μM
TBO 2 μM
Kleb. pneumonia, Clinical resistant isolate
TBO 0 μM
TBO 2 μM
TBO 3 μM
TBO 5 μM
Ps. aeruginosa, ATCC 27853
TBO 0 μM
TBO 2.5μM
TBO 5μM
TBO 10μM
Cadida glabrate, ATCC 90030
Survival Fraction
0 uM
25 uM
50 uM
100 uM
Cadida krusei, ATCC 6258
0 uM
10 uM
20 uM
40 uM
Ps. aeruginosa, Clinical resistant isolate
TBO 0 μM
TBO 2.5μM
TBO 5μM
TBO 10μM
Cadida glabrate, Clinical resistant isolate
0 uM
25 uM
50 uM
100 uM
Cadida krusei, Clinical resistant isolate
0 uM
10 uM
20 uM
40 uM



the strains measured in the eight tested strains using flow cytometry. There was no significant PpIX accumulation in either the reference strain or the clinical isolates for all the tested pathogens (data not shown).

All three tested PSs (at 200 μM MB, 10 μM TBO and 3 μM ALA) were able to reduce the LPS level after PDI using LAL assay at the sensitivity limits of 0.25 and 0.03 EU/mL.

Discussion

This *in vitro* study showed the photo-antimicrobial efficacy of MB, TBO and ALA against eight bacterial suspensions (reference strains and clinical wild-type and MDR strains) and four fungal strains using different PDI doses. The killing effect of PDI depends on the generation of reactive oxygen species. Further experiments are required to confirm the PDI effect and more wild type and MDR strains should be included. Moreover, application of the PS on cell surfaces should be investigated as this will provide valuable information in the clinical setting. With appropriate selection and improvement of PS, PDI may become a useful therapeutic adjunct to address the antibiotic resistance and MDR problem.

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