

RESEARCH PAPER

## Antimicrobial blue light inactivation of *Candida albicans*: *In vitro* and *in vivo* studies

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### ABSTRACT

Fungal infections are a common cause of morbidity, mortality and cost in critical care populations. The increasing emergence of antimicrobial resistance necessitates the development of new therapeutic approaches for fungal infections. In the present study, we investigated the effectiveness of an innovative approach, antimicrobial blue light (aBL), for inactivation of *Candida albicans* *in vitro* and in infected mouse burns. A bioluminescent strain of *C. albicans* was used. The susceptibilities to aBL (415 nm) were compared between *C. albicans* and human keratinocytes. The potential development of aBL resistance by *C. albicans* was investigated via 10 serial passages of *C. albicans* on aBL exposure. For the animal study, a mouse model of thermal burn infected with the bioluminescent *C. albicans* strain was used. aBL was delivered to mouse burns approximately 12 h after fungal inoculation. Bioluminescence imaging was performed to monitor in real time the extent of infection in mice. The results obtained from the studies demonstrated that *C. albicans* was approximately 42-fold more susceptible to aBL than human keratinocytes. Serial passaging of *C. albicans* on aBL exposure implied a tendency of reduced aBL susceptibility of *C. albicans* with increasing numbers of passages; however, no statistically significant difference was observed in the post-aBL survival rate of *C. albicans* between the first and the last passage ( $P > 0.05$ ). A single exposure of 432 J/cm<sup>2</sup> aBL reduced the fungal burden in infected mouse burns by 1.75- $\log_{10}$  ( $P = 0.015$ ). Taken together, our findings suggest aBL is a potential therapeutic for *C. albicans* infections.

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## Introduction

Fungal infections, including fungal burn or wound infections, are a common cause of morbidity, mortality and cost in critical care populations.<sup>1–3</sup> It is estimated that fungal infections occur in over a billion people each year, and recent evidence suggests the rate is increasing.<sup>4</sup> *Candida* spp. are the most common causative species of fungal infections.<sup>5,6</sup> Among *Candida* spp, *Candida albicans* is the major global pathogen, causing 50%–70% of candidiasis cases.<sup>7</sup> The increasing emergence of antimicrobial drug resistance has significantly compromised the management of fungal infections.<sup>8–11</sup> Recently, resistance to azole drugs, the most used class of antifungals, has been identified in *C. albicans*.<sup>12–14</sup> As a result, there is a

pressing need for the development of new approaches to tackle drug resistance in fungal infections.<sup>8</sup>

As a non-antibiotic approach, light-based antimicrobial therapies, including antimicrobial photodynamic therapy (aPDT) and ultraviolet-C (UVC) irradiation, have been extensively investigated as alternative therapeutics for infectious diseases, especially for localized infections.<sup>15,16</sup> Advantages of light-based therapies include rapid action and equal inactivation effectiveness regardless of drug resistance.<sup>17–19</sup> However, one major disadvantage of aPDT, as a two-part (photosensitizer + light) combination approach, is the challenge of introducing exogenous photosensitizers into certain pathogens and less than perfect selectivity for pathogens over

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host cells.<sup>20</sup> The use of UVC, on the other hand, has limitations due to its detrimental effects on host cells.<sup>16</sup>

An innovative light-based antimicrobial approach, antimicrobial blue light (aBL), has attracted increasing attention due to its intrinsic ability to inactivate pathogens without the involvement of exogenous photosensitizers.<sup>21-23</sup> The mechanism of action of aBL is still not fully understood. A common hypothesis is that aBL excites naturally occurring endogenous photosensitizers in the cells of pathogens and subsequently leads to the production of cytotoxic oxidative species.<sup>21,24</sup> However, the use of aBL for treatment of actual infections has yet to become established.<sup>21,22</sup> The majority of the publications on aBL have been confined to *in vitro* efficacy studies.<sup>25-38</sup> There have been only three published reports to demonstrate the efficacy of aBL for infections *in vivo*.<sup>39-41</sup> It has been demonstrated that aBL (415-nm) significantly reduced the bacterial burden (both Gram-positive and Gram-negative) in mouse wounds or burns,<sup>39-41</sup> and was protective in a lethal mouse model of *P. aeruginosa* infection.<sup>40</sup>

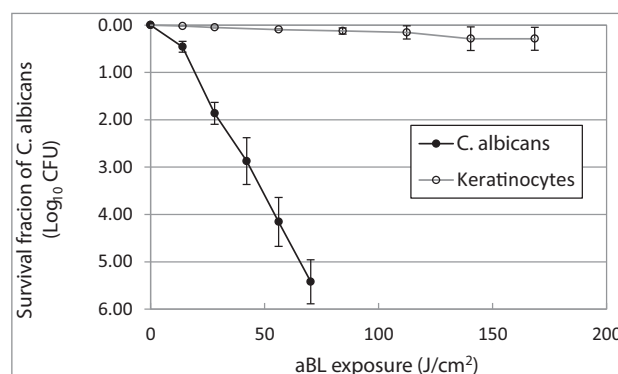
In the present study, we investigated for the first time the use of aBL for *C. albicans* infections in mouse burns. Burns provide an ideal portal for invasive infections while also inducing substantial immune dysfunction; as a result, burn patients are cited as being among the highest risk groups for invasive fungal infections.<sup>3</sup> The results obtained from the present study demonstrated that *C. albicans* was much more susceptible to aBL than human keratinocytes. Serial passaging of *C. albicans* on aBL exposure implied a tendency of reduced aBL susceptibility of *C. albicans* with increasing numbers of passages; however, no statistically significant difference was observed in the post-aBL survival rate of *C. albicans* between the first and the last passage. A single exposure of aBL (432 J/cm<sup>2</sup>) significantly reduced the fungal burden in infected mouse burns.

## Results

### Susceptibility to aBL: *C. albicans* vs. human keratinocytes

To ensure a selective aBL inactivation of fungal cells over host cells, *in vitro* study was conducted to compare aBL susceptibilities between *C. albicans* and human keratinocytes. The results of keratinocytes were obtained from our previous study,<sup>41</sup> in which human keratinocytes (HaCaT) were exposed to aBL under the equivalent condition to that for aBL inactivation of *C. albicans* in the present study.

The aBL inactivation curves of both *C. albicans* and keratinocytes approximately followed the first-order kinetics, showing a linear relation between the log-



**Figure 1.** Antimicrobial blue light inactivation of *C. albicans* and human keratinocytes *in vitro*. Bars: standard deviation. The mean inactivation rate coefficients ( $k_H$ ) of *C. albicans* and keratinocytes were 0.0795 and 0.0019 cm<sup>2</sup>/J, respectively, indicating an approximately 42-fold faster inactivation rate of *C. albicans* by aBL than keratinocytes.

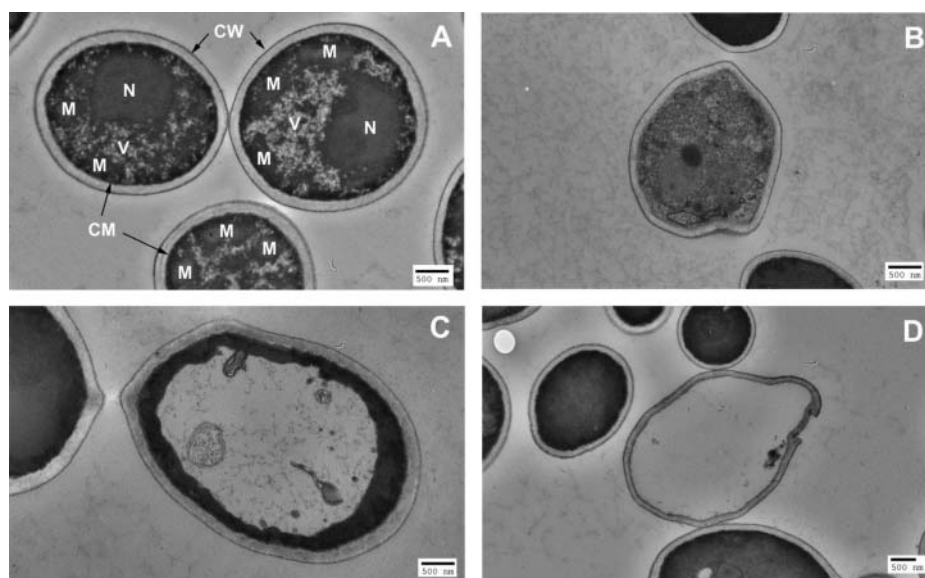
transformed cell survival fraction log<sub>10</sub> and aBL radiant exposure (Fig. 1).<sup>42</sup> It can be seen that *C. albicans* cells are much more susceptible to aBL than keratinocytes. When an exposure of 70.2 J/cm<sup>2</sup> light had been delivered, 5.42-log<sub>10</sub> colony forming units (CFU) of *C. albicans* were inactivated on average, while only 0.11-log<sub>10</sub> viability loss in keratinocytes was observed under the equivalent condition. The mean inactivation rate coefficients ( $k_H$ )<sup>43</sup> of *C. albicans* and keratinocytes, which were estimated from the slopes of the inactivation curves, were 0.0795 and 0.0019 cm<sup>2</sup>/J, respectively, indicating an approximately 42-fold faster inactivation rate of *C. albicans* by aBL than keratinocytes.

### Transmission electron microscopy (TEM) imaging of *C. albicans* cells

Under TEM, untreated *C. albicans* cells showed typical morphology of *C. albicans* containing a nucleus, vacuoles and mitochondria, surrounded by cytoplasmic membrane and cell wall (Fig. 2A). When an exposure of 35.1 J/cm<sup>2</sup> aBL had been delivered, disruption of inner organelles in some *C. albicans* cells with a deformed cell wall exhibited (Fig. 2B). In some cases, unusual vacuole morphology was observed, driving electron-dense structures to the cell periphery (Fig. 2C). When an exposure of 70.2 J/cm<sup>2</sup> light had been delivered, complete decomposition of inner organelles with disrupted cell walls and almost complete loss of cytoplasmic components were seen in some *C. albicans* cells (Fig. 2D).

### Fluorescence spectroscopic measurements

We investigated the characteristic fluorescence emission spectra of *C. albicans* upon excitation at defined absorption peaks by using *C. albicans* cell lysates. Excitation at



**Figure 2.** TEM images of *C. albicans* cells. (A) Untreated *C. albicans* cells: N=nucleus; V = vacuole; M=mitochondria; CM = cytoplasmic membrane; CW = cell wall. (B)-(D) aBL-treated *C. albicans* cells: (B) Decomposition of inner organelles with deformed cell wall (aBL radiant exposure = 35.1 J/cm<sup>2</sup>); (C) Unusual vacuole growth, driving electron-dense structures to the cell periphery (aBL radiant exposure = 35.1 J/cm<sup>2</sup>); and (D) Complete loss of cytoplasmic components with disrupted cell wall (aBL radiant exposure = 70.2 J/cm<sup>2</sup>).

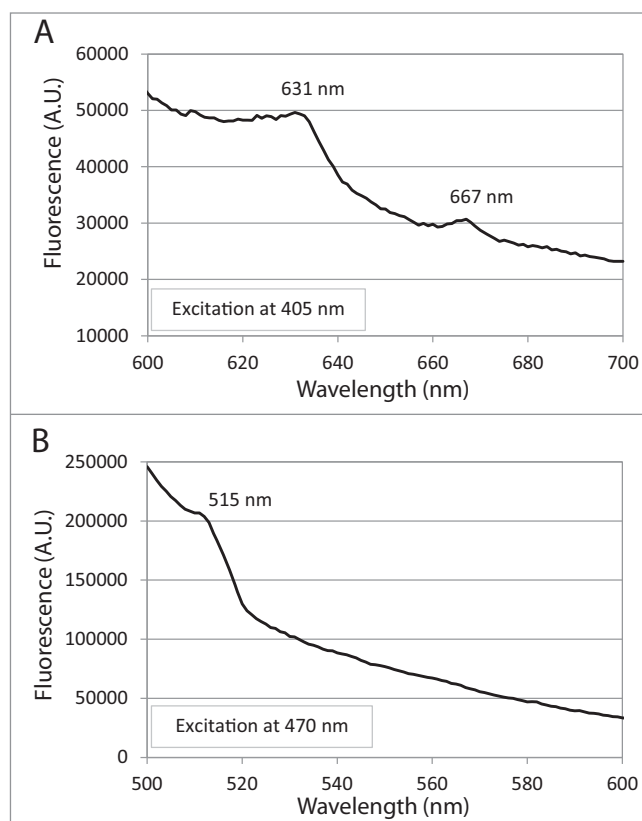
405-nm, which is the absorption peak of porphyrin-like compounds, led to porphyrin-like dual fluorescence emission maxima at 631 and 667 nm (Fig. 3A);<sup>44</sup> while excitation at 470 nm, which is the absorption peak of flavin-like compounds, gave rise to a flavin-like emission maximum at 515 nm (Fig. 3B).<sup>45</sup>

#### Serial passaging for *C. albicans* on aBL exposure

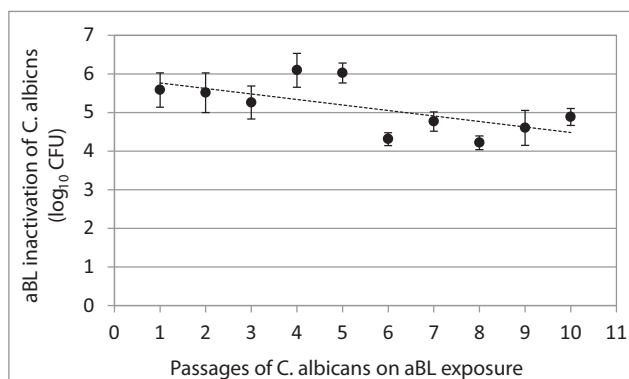
*C. albicans* suspensions were serially passaged 10 times on aBL exposure, and Figure 4 shows the changes in the extent of aBL inactivation of *C. albicans* with increasing numbers of passages. A correlation analysis indicated a tendency of decrease in aBL susceptibility with the numbers of passages (correlation coefficient = -0.64;  $P=0.045$ ). However, statistical analysis revealed no significant difference in aBL inactivation extent between the 1st and 10th passage ( $P=0.09$ ).

#### aBL therapy for *C. albicans* infection in mouse burns

Our results indicated that aBL significantly reduced fungal burden in infected mouse burns. Figure 5A shows a set of bioluminescence images of a representative mouse burn infected with 10<sup>5</sup> CFU of *C. albicans* and treated with a single exposure of 432 J/cm<sup>2</sup> aBL. aBL was delivered at 12 h after fungal inoculation. Immediately after aBL exposure, only a few pixels of remaining fungal luminescence were observed. Although recurrence of fungal luminescence (indicating infection regrowth) was observed during the following days in the aBL-treated



**Figure 3.** Fluorescence emission spectra of lysed *C. albicans* cells in NaOH/SDS: (A) Excitation at 405 nm led to porphyrin-like dual fluorescence emission maxima at 631 and 667 nm; (B) Excitation at 470 nm gave rise to a flavin-like emission maximum at 515 nm.



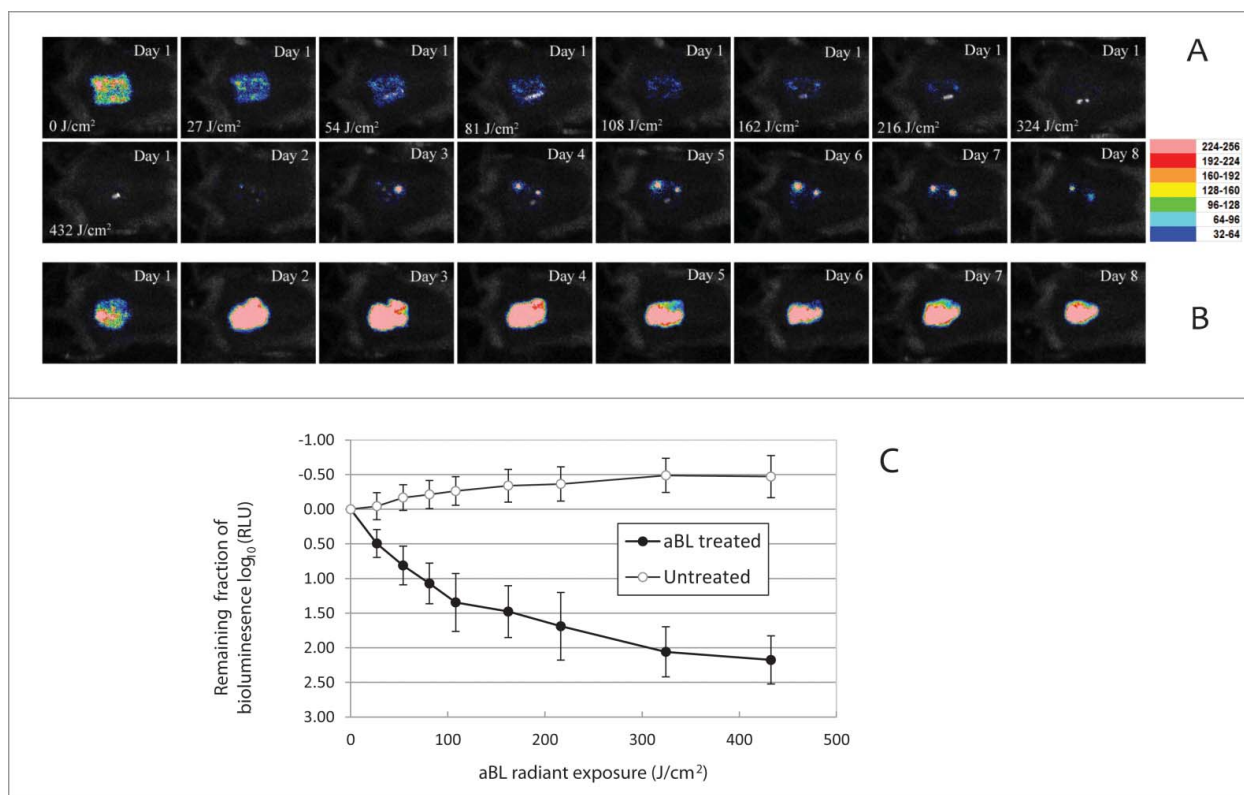
**Figure 4.** Change of extent of aBL inactivation of *C. albicans* (log<sub>10</sub> CFU) with numbers of passages of *C. albicans* on aBL exposure. Bars: standard deviation. No statistically significant difference was observed in aBL inactivation extent between the 1st and 10th passage ( $P = 0.09$ ).

mouse burn, the extent of infection (or fungal load) in the untreated mouse burn was always more than 1-log<sub>10</sub> unit (10 fold) higher than that of the aBL-treated mouse burn during the whole period of experiment (at all the

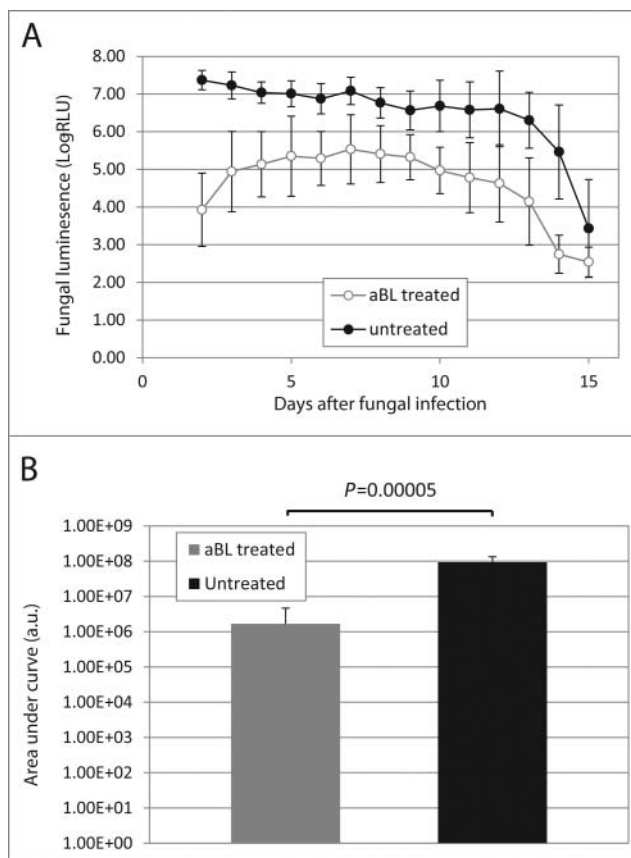
time points sampled). It can also be seen from Figure 5B that, in the untreated mouse burn, infection steadily developed with time and remained strong until 8 days after fungal inoculation. (Fig. 5B)

Quantitative analysis of the results supported the above findings from bioluminescence images. Figure 5C shows the dose-response curves of mean fungal luminescence (relative light units: RLU) from a group of mouse burns ( $n = 8$ ) infected with  $10^5$  CFU *C. albicans* and treated with aBL at 12 h after fungal inoculation. Approximately 2.18-log<sub>10</sub> (99.3%) inactivation of *C. albicans* in mouse burns was achieved after an exposure of 432 J/cm<sup>2</sup> aBL had been delivered. The fungal luminescence of the mouse burns without being exposed to aBL ( $n = 8$ ) increased by 0.39-log<sub>10</sub> during the equivalent period of time ( $P < 0.0001$ ).

Figure 6A shows the time courses of the mean fungal luminescence (RLU) from day 2 (the next day after aBL therapy) to day 15 of mouse burns infected with  $10^5$  CFU of *C. albicans* followed by treatment with a single exposure of 432 J/cm<sup>2</sup> aBL delivered at 12 h



**Figure 5.** (A-B) Successive fungal luminescence images of representative mouse burns infected with  $10^5$  CFU of bioluminescent *C. albicans*, with (panel A) and without aBL therapy (panel B), respectively. aBL irradiance = 90 mW/cm<sup>2</sup>. aBL was delivered at 12 h after fungal inoculation. In panel A, the Day 1-0 J/cm<sup>2</sup> image was taken just prior to aBL exposure; Day 1-27 J/cm<sup>2</sup>, Day 1-54 J/cm<sup>2</sup>, Day 1-81 J/cm<sup>2</sup>, Day 1-108 J/cm<sup>2</sup>, Day 1-162 J/cm<sup>2</sup>, Day 1-216 J/cm<sup>2</sup>, Day 1-324 J/cm<sup>2</sup> and Day 1-432 J/cm<sup>2</sup> images were taken immediately after respective aBL exposure had been delivered; and the Day 1 to Day 8 images were taken at Day 1 to Day 8 after fungal inoculation, respectively. In panel B, the Day 1 to Day 8 images were taken at Day 1 to Day 8 after fungal inoculation, respectively. (C) Dose responses of mean fungal luminescence of mouse burns infected with  $10^5$  CFU of *C. albicans*, with ( $n = 8$ ) and without ( $n = 8$ ) aBL therapy, respectively. aBL was delivered at 12 h after fungal inoculation. Bars: standard deviation.



**Figure 6.** (A) Time courses of mean fungal luminescence (RLU, day 2 to day 11) of mouse burns infected with  $10^5$  CFU of *C. albicans*, with ( $n=8$ ) and without ( $n=8$ ) aBL therapy, respectively. aBL was delivered at 12 h after fungal inoculation. Bars: standard deviation. (B) Mean areas under the fungal luminescence versus time curves in the two-dimensional coordinate system in panel A, representing the overall fungal burden of infected mouse burns. Bars: standard deviation.

post-inoculation ( $n = 8$ ) as well as infected mouse burns without aBL ( $n = 8$ ). In the untreated mice, fungal luminescence remained strong until day 12. In both the aBL-treated and untreated mice, burn scabs started to peel off from the backs of mice as of approximately day 12. Very modest fungal luminescence remained in the burns after the scabs peeled off. Statistical analysis of the areas under the curves (AUC) of bioluminescence time course in the two-dimensional coordinate system in Figure 6A demonstrated that aBL significantly decreased fungal burden of the infected burns (Fig. 6B). The mean values of AUC of aBL-treated and untreated mice were  $1.67 \times 10^6$  and  $9.40 \times 10^7$  ( $P = 0.00005$ ), respectively, indicating a 1.75- $\log_{10}$  (98.2%) reduction of fungal burden in mouse burns as a result of aBL therapy.

## Discussion

*Candida*, a fungus that commonly causes serious illness, especially among hospital patients, is showing increasing

resistance to the drugs used for treatment.<sup>8-11</sup> This fact underlines the need for new treatments against *Candida* infections.<sup>8</sup> In the present study, we investigated for the first time an innovative light-based approach, aBL, for treatment of *C. albicans* infections in mouse models. The study may serve as an initial effort in the pursuit of utilizing aBL therapy for fungal infections, especially those caused by drug-resistant strains. Thus, the first and most important impact of the study is the opening of an emerging area of study using a different therapeutic regimen for fungal infections. The results of the present study demonstrated that *C. albicans* was much more susceptible to aBL than human keratinocytes. Serial passaging of *C. albicans* on aBL exposure implied a tendency of reduced aBL susceptibility of *C. albicans* with increasing numbers of passages; however, no statistically significant difference was observed in the post-aBL survival rate of *C. albicans* between the first and the last passage. A single exposure of aBL ( $432 \text{ J/cm}^2$ ) significantly reduced the fungal burden in infected mouse burns.

The results of the *in vitro* susceptibility study indicate that there exists a therapeutic window where *C. albicans* cells are selectively inactivated by aBL while host cells can be preserved. However, only one *C. albicans* strain and human keratinocytes were investigated in the present study. To confirm this finding, more *C. albicans* strains as well as other fungal species should be tested in the future to compare their aBL susceptibilities with those of host cells other than keratinocytes (i.e., fibroblasts, myoblasts, endothelial cells, etc.).

Fluorescence spectroscopic measurements suggested that *C. albicans* contains both endogenous porphyrins and flavins. In this study, aBL at 415 nm was delivered for inactivation of *C. albicans* both *in vitro* and *in vivo*. As 415-nm wavelength is close to the absorption peak of porphyrins (405 nm), it supports the hypothesis that the inactivation of *C. albicans* was due to the photo-excitation of endogenous porphyrins. On the other hand, the presence of flavins in *C. albicans* implies that *C. albicans* may also be susceptible to aBL at 470 nm. This is supported by a previous study showing aBL at 470 nm is fungicidal without the involvement of exogenous photosensitizers.<sup>46</sup> Taken together, fluorescence spectroscopic measurements supported the hypothesis that endogenous porphyrins and/or flavins are associated with aBL inactivation of *C. albicans*. To further relate the action of aBL toxicity and porphyrins/flavins presence, future studies are needed to include the results from *C. albicans* strains deprived of the production of porphyrins and/or flavins. In addition, for better characterization of these endogenous photosensitizing substances, high performance liquid chromatography

(HPLC) measurements should be performed in future studies.

To use aBL for inactivation of pathogens, one question that will have to be addressed is “Can pathogens develop aBL resistance?” An interesting finding in a previous study of our laboratory showed that a multidrug-resistant *A. baumannii* strain failed to develop aBL resistance after 10 serial passages on aBL exposure.<sup>41</sup> On the contrary, there was a tendency of increased aBL susceptibility of *A. baumannii* with increasing numbers of passages.<sup>41</sup> In the present study, we investigated the potential development of aBL resistance by *C. albicans* also by carrying out 10 serial passages of *C. albicans* on aBL exposure. Correlation analysis indicated a tendency for aBL susceptibility of *C. albicans* to decrease with increasing numbers of passages. However, statistical analysis did not show a significant difference in aBL inactivation extent of *C. albicans* between the 1st passage and the last (10<sup>th</sup>) passage. Further study (e.g., increase the number of passages of *C. albicans* on aBL exposure, i.e., up to 80 passages<sup>47</sup>) are imperative to investigate the aBL resistance development and, if aBL resistance does develop, to elucidate the molecular mechanism of reduced aBL susceptibility of *C. albicans* after repeated aBL inactivation.

The *in vivo* study demonstrated that a single exposure of aBL at 432 J/cm<sup>2</sup> significantly reduced fungal burden in mouse burns in comparison to the untreated burns. Modest to mild recurrence of fungal luminescence (infection) was observed in some aBL-treated mouse burns. Future study will be carried out to counteract the recurrence of infection by, e.g., repeated aBL therapy.

A pitfall of the present study is that the effect of melanization by *C. albicans* during the infection was not investigated. *C. albicans* and many other fungi can produce melanin during infection. Melanin absorbs light and, subsequently, protects the fungi from being inactivated by aBL. In the present study, *C. albicans* cells were not grown in melanin inducing conditions. Future study is warranted to test the effects of melanization on aBL efficacy.

Another limitation of the present study lies in the fact that the mouse infection model used only represents the scenario of superficial infections. For deeply seated infections, topical application of aBL may not be able to reach the sites of infection. We are now investigating interstitial light delivery to treat deeply seated infections by using an optical needle array.<sup>48</sup>

Taken together, one can postulate that aBL is a potential alternative therapeutics for fungal infections. Future work is still needed to optimize this approach and introduce it to clinic.

## Material and methods

### Blue light source

In the cell culture study, aBL was delivered using an Omnilux clear-U<sup>TM</sup> light emitting diode (LED) array (Photo Therapeutics, Inc., Carlsbad, CA) with a central wavelength of 415-nm and a full-width half maximum of 20 nm. In the animal study, to avoid prolonged light exposure time, we used a more powerful prototype LED (Vielight Inc., Toronto, Canada) with the peak emission at 415 nm and the full-width half maximum of 10 nm. Irradiance of aBL was measured using a PM100D power/energy meter (Thorlabs, Inc., Newton, NJ).

### Bioluminescent *C. albicans* strain and culture condition

The bioluminescent *C. albicans* strain used in the study was CEC 749, as described previously.<sup>49</sup> In brief, the luciferase reporter was constructed by fusing a synthetic, codon-optimized version of the *Gaussia princeps* luciferase gene to *C. albicans* PGA59, which encodes a glycosyl-phosphatidylinositol-linked cell wall protein. Luciferase expressed from this PGA59-gLUC fusion was localized at the *C. albicans* cell surface,<sup>49</sup> allowing the detection of luciferase in intact cells after the addition of the luciferase substrate, coelenterazine.

*C. albicans* was routinely grown at 30°C on yeast peptone dextrose (YPD) agar and sub-cultured in YPD medium to an optical density of 0.65 at 570 nm, which corresponds to 10<sup>7</sup> CFU/mL. The suspension was then centrifuged, washed with phosphate-buffered saline (PBS), and re-suspended in PBS at the cell density of 10<sup>7</sup> CFU/mL again.

### aBL inactivation of *C. albicans* *in vitro*

Three (3) mL of *C. albicans* suspension in PBS at a cell density of ≈10<sup>7</sup> CFU/mL was placed into a 35-mm petri dish at room temperature (21°C). The suspension was exposed to aBL at an irradiance of 19.5 mW/cm<sup>2</sup> (the maximum irradiance of this light source) with the lid of the Petri dish removed. During light exposure, the *C. albicans* suspension was gently stirred by a mini-magnetic bar (Fisher Scientific Co., Norcross, GA) at 20 rpm. Thirty (30) μL aliquots of the suspension were withdrawn at 0, 12, 24, 36, 48, and 60 min, respectively, when 0, 14.0, 28.1, 42.1, 56.2, and 70.2 J/cm<sup>2</sup> light had been delivered. CFU were then determined by serial dilutions on YPD agar plates.<sup>50</sup> Colonies were allowed to grow for 18-24 h at 30°C. The experiments were performed in triplicate.

### Transmission electron microscopy (TEM)

aBL-treated or untreated *C. albicans* cells were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde mixture immediately after aBL exposure and stored for 24 h at 4°C. After centrifuge (1200 rpm, 10 min) and decanting the fixative, 0.1 M sodium cacodylate buffer (pH 7.2) was added to the pellets. After fixation, hot agar (2% in distilled water, heated to boiling) was immediately added to each pellet. Once the agar had hardly solidified, the cell pellets were then processed routinely for transmission electron microscope. The cell pellets were postfixed in 2% OsO<sub>4</sub> in sodium cacodylate buffer, dehydrated in a graded alcohol series, and embedded in Epon t812 (Tousimis, Rockville, MD). Ultrathin sections (80–100 nm thick) were cut on a Reichert-Jung Ultracut E microtome (Vienna, Austria), collected on uncoated 200 mesh copper grids, stained with uranyl acetate (2 min) and lead citrate (2 min), and examined on a Philips CM-10 transmission electron microscope (Eindhoven, The Netherlands). The negatives were scanned on an Epson Perfection 3200 photo-scanner. Multiple parasite sections were microscopically analyzed and images representing the most typically observed morphologies were presented in the study.

### Cell lysis of *C. albicans* and fluorescence spectroscopic measurement

To identify the presence and types of endogenous photosensitizers, *C. albicans* cells were lysed to investigate their fluorescence-emission characteristics.<sup>32</sup> Briefly, an overnight *C. albicans* culture (3 mL) was centrifuged, washed with PBS, centrifuged again, and the supernatant removed. The *C. albicans* cells were then lysed by re-suspending the *C. albicans* pellets in 1 mL of a mixture of 0.1 M NaOH/1% sodium dodecyl sulfate (SDS), and the suspension was allowed to stand in the dark for 24 h. Fluorescence of the *C. albicans* lysates in NaOH/SDS (in a cuvette 1 cm thick) was measured on a fluorimeter (Fluoromax 3, SPEX Industries, Edison, NJ), with excitation at 405 or 470 nm and emission scanned from 580 to 700 nm.

### Serial passaging of *C. albicans* on aBL exposure

To investigate the potential development of aBL resistance by *C. albicans*, *C. albicans* suspension was subjected to 10 serial passages on aBL exposure. In each passage, three independent cultures were tested. For each culture, 3 mL *C. albicans* suspension containing 10<sup>7</sup> CFU/mL in PBS was placed into a 35-mm petri dish. The suspension was then exposed to

aBL at the irradiance of 19.5 mW/cm<sup>2</sup>. During aBL exposure, the *C. albicans* suspension was gently stirred with a miniature magnetic bar (20 rpm). In the 1st passage, the radiant exposure of aBL was adjusted to leave about 0.0004% fungal cells surviving (5.42-log<sub>10</sub> inactivation) after aBL, and the same radiant exposure level of aBL was then used throughout the successive passages. Fungal CFU was determined by serial dilutions on YPD agar plates.<sup>50</sup> The surviving fungal cells after aBL exposure were re-cultured for the next passage. This procedure was repeated until the 10th passage was reached. Fungal survival rates in different passages were compared using an one-way ANOVA.

### Animals

Adult female BALB/c mice (Charles River Laboratories, Wilmington, MA), 6–8 week old and weighing 17–21 g, were used. Mice were given access to food and water *ad libitum*, and maintained on a 12-hour light/dark cycle under a room temperature of 21°C. All animal procedures in this study were approved by the Subcommittee on Research Animal Care (IACUC) of Massachusetts General Hospital and were in accordance with the guidelines of the National Institutes of Health (NIH).

### *C. albicans* infection in acute mouse burns

Before the infliction of burns, mice were anesthetized by intraperitoneal (I.P.) injection of a ketamine-xylazine cocktail and then shaved on the dorsal surfaces. Burns were inflicted by applying a preheated (≈95°C) brass block (Small Parts, Inc., Miami, FL) to the dorsal surfaces of mice for 3 s, resulting in nonlethal and third-degree thermal injuries. The brass block area was 12 mm × 12 mm, corresponding to 3.6% of the total body surface area<sup>51</sup> of mice. Immediately after burn injuries, mice were resuscitated with I.P. injections of 0.5 mL sterile saline to prevent dehydration.

Five (5) min after the infliction of burns (to allow the burns to cool down), a 50-μL aliquot of *C. albicans* in sterile PBS containing 10<sup>5</sup> cells was inoculated to the eschar of each mouse burn with a pipette tip and was then smeared onto the eschar with an inoculating loop. Mice were imaged with a bioluminescence camera immediately after the inoculation of *C. albicans* to ensure that the fungal inoculum applied to each mouse burn was consistent. Our previous studies showed that both conidia and hyphae were presented in the mouse wounds when infections established.<sup>52,53</sup>

## **aBL inactivation of *C. albicans* in infected mouse burns**

Sixteen (16) mice with infected burns were randomly divided into 2 groups of 8 mice each: one group was exposed to aBL and the other not (untreated controls). aBL was delivered at approximately 12 h (overnight) after fungal inoculation. Mice were given a total radiant exposure of up to 432 J/cm<sup>2</sup> (80 min exposure time at the irradiance of 90 mW/cm<sup>2</sup>). To record the time course of the extent of infection, fungal luminescence of mouse burns was measured daily after aBL exposure until the infections resolved (characterized by the disappearance of fungal luminescence) or for up to 15 days after aBL therapy.

## **Bioluminescence imaging**

We used an *in vivo* bioluminescence imaging technique to noninvasively monitor in real time the extent of infection in living mice.<sup>54</sup> Our previous study demonstrated a perfectly linear correlation between *C. albicans* luminescence readout (RLU) and the corresponding CFU.<sup>52,53</sup> This method is a significant improvement on the traditional use of animal survival or body fluid sampling and subsequent plating and colony counting. The setup consists of an ICCD camera (C2400-30H, Hamamatsu Photonics, Bridgewater, NJ), a camera controller, a specimen chamber, an image processor (C5510-50, Hamamatsu), and a color monitor (PVM 1454Q, Hamamatsu). White light LEDs are mounted inside the specimen chamber and supply the light required for obtaining dimensional imaging of the sample. Under photo-counting mode, a clear image can be obtained even under extremely low-light levels by detecting and integrating individual photons one by one.

Prior to imaging, mice were anesthetized by I.P. injections of a ketamine/xylazine cocktail. Twenty (20)  $\mu$ L coelenterazine (Gold Biotechnology, Inc., St. Louis, MO; 500  $\mu$ g/mL in 1:9 methanol-PBS) was topically applied to the eschars of infected burns. Mice were then placed on a height-adjustable stage in the specimen chamber, and the infected mouse burns were positioned directly under the camera. A gray-scale background image of each mouse burn was made, and this was followed by a photon count of the same region. This entire burn photon count was quantified as RLU and was displayed in a false color scale ranging from pink (most intense) to blue (least intense).

## **Statistical analysis**

Data were expressed as the mean  $\pm$  SD. Differences were tested for significance using a one-way ANOVA. Values of  $P < .05$  were considered statistically significant.

## **Disclosure of potential conflicts of interest**

Y Zhang, Y Zhu, JC, YW, MES, MSV, DCH, MRH and TD declare that they have no conflicts of interest. CKM is an employee of the US government. The views expressed herein are those of the author and do not reflect the official policy or position of the Department of the Army, Department of Defense, or the US Government. This work was prepared as part of his official duties and, as such, there is no copyright to be transferred.

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Research was conducted in compliance with the Animal Welfare Act Regulations and other Federal statutes relating to animals and experiments involving animals and adheres to the principles set forth in the Guide for Care and Use of Laboratory Animals, National Research Council, 1996.

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