Dual-wavelength photo-killing of methicillin resistant Staphylococcus aureus

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ABSTRACT With the effectiveness of antimicrobials declining as antimicrobial resistance continues to threaten public health, we must look to alternative strategies for the treatment of infections. In this study, we investigated an innovative ‘drug-free’ dual-wavelength irradiation approach that combines two wavelengths of light, 460 nm and 405 nm, against methicillin resistant Staphylococcus aureus (MRSA). MRSA was initially irradiated with 460 nm light (90-360 J/cm²) and subsequently irradiated with aliquots of 405 nm light (54-324 J/cm²). For in vivo studies, mouse skin was abraded and infected with approximately 10^7 CFU of MRSA and incubated for 3 hours before irradiating with 460 nm (360 J/cm²) and 405 nm (342 J/cm²). Naïve mouse skin was also irradiated to investigate apoptosis. We found that staphyloxanthin, the carotenoid pigment in MRSA cells, promoted resistance to the antimicrobial effects of 405 nm light. In addition, we found that the photolytic effect of 460 nm light on staphyloxanthin attenuated resistance of MRSA to 405 nm light inactivation. Irradiation of 460 nm alone did not elicit any antimicrobial effect on MRSA. In a proof-of-principle mouse skin abrasion infection model, we observed significant inactivation of MRSA by the dual-wavelength irradiation approach. However, when either wavelength of light was administered alone, no significant decrease in bacterial viability was observed. Moreover, exposure of the dual-wavelength irradiation to naïve mouse skin did not […]

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With the effectiveness of antimicrobials declining as antimicrobial resistance continues to threaten public health, we must look to alternative strategies for the treatment of infections. In this study, we investigated an innovative ‘drug-free’ dual-wavelength irradiation approach that combines two wavelengths of light, 460 nm and 405 nm, against methicillin resistant *Staphylococcus aureus* (MRSA). MRSA was initially irradiated with 460 nm light (90-360 J/cm²) and subsequently irradiated with aliquots of 405 nm light (54-324 J/cm²). For *in vivo* studies, mouse skin was abraded and infected with approximately $10^7$ CFU of MRSA and incubated for 3 hours before irradiating with 460 nm (360 J/cm²) and 405 nm (342 J/cm²). Naïve mouse skin was also irradiated to investigate apoptosis. We found that staphyloxanthin, the carotenoid pigment in MRSA cells, promoted resistance to the antimicrobial effects of 405 nm light. In addition, we found that the photolytic effect of 460 nm light on staphyloxanthin attenuated resistance of MRSA to 405 nm light killing. Irradiation of 460 nm alone did not elicit any antimicrobial effect on MRSA. In a proof-of-principle mouse skin abrasion infection model, we observed significant killing of MRSA by the dual-wavelength irradiation approach. However, when either wavelength of light was administered alone, no significant decrease in bacterial viability was observed. Moreover, exposure of the dual-wavelength irradiation to naïve mouse skin did not result in any visible apoptosis. In conclusion, dual-wavelength irradiation strategy may offer an innovative, effective and safe approach for the treatment of skin infections caused by MRSA.
In recent years, the threat of antimicrobial resistance has become one of the most important concerns to public health. Infection outbreaks that result from multidrug resistant organisms that have emerged remains a significant problem\textsuperscript{1,2}. *Staphylococcus aureus* infections are amongst the most important threats that can result in skin and soft-tissue infections, with methicillin resistant *Staphylococcus aureus* (MRSA) being particularly important\textsuperscript{3,4}. Therefore, novel, non-traditional approaches must be explored to quell these negative effects. Over the years, antimicrobial blue light (aBL) at 405 nm wavelength has been emerging as a potential alternative treatment for localized infections\textsuperscript{5}. The accepted mechanism responsible for the antimicrobial effects of aBL (405 nm) is through excitation of endogenous photosensitizing porphyrins and the subsequent generation of singlet oxygen resulting in lipid peroxidation, DNA damage, cell wall damage and cellular apoptosis of microbial cells\textsuperscript{5}. However, previous studies and the preliminary results in our laboratory showed that MRSA is much more tolerant of aBL at 405 nm than most of other species\textsuperscript{6}. Recent findings by other groups have demonstrated the antioxidant properties of staphyloxanthin (STX), which is a membrane-bound carotenoid pigment of MRSA, responsible for its characteristic golden colonial phenotype\textsuperscript{7,8}. In addition, it has been demonstrated that STX is subject to photolysis through 460 nm light exposure, rendering it more susceptible to H\textsubscript{2}O\textsubscript{2} mediated killing\textsuperscript{9}. Therefore, we theorized that the limited antimicrobial efficacy of aBL (405 nm) we have observed with MRSA was a direct result of STX, as this is too a known singlet oxygen scavenger. As a result, we hypothesized that treatment of MRSA using 405 nm light can be improved by STX photolysis using pre-460 nm light illumination. Here we report a novel, dual-wavelength irradiation approach, using the combination of 460 nm and 405 nm light, that exploits the STX photolytic effect of 460 nm light to sensitize MRSA to 405 nm light.
RESULTS

STX promoted resistance in *S. aureus* to 405 nm light mediated killing

In this study, we investigated whether the presence of STX within MRSA is responsible for its inherent resistance to aBL (405 nm). We compared the killing potential of a wild-type (WT) *S. aureus* strain (Pig1) that produces STX and an ΔcrtM isogenic mutant (Pig1::ΔcrtM) of the same strain that is deficient in the production of STX. Following 108 J/cm\(^2\) aBL, a 4.14 log\(_{10}\) CFU reduction was achieved in Pig1::ΔcrtM, compared with its WT parental strain in which only 1.09 log\(_{10}\) CFU reduction was observed (*P*<0.001; Figure 1). Findings thus demonstrate the ability for STX to promote resistance to aBL at 405 nm.

Exposure to 460 nm light sensitized MRSA to 405 nm light

A recent study discovered that 460 nm irradiation can lyse the STX pigment, rendering MRSA more sensitive to ROS-mediated killing\(^9\). Therefore, we sought to determine whether pre-irradiation with 460 nm light was able to attenuate STX mediated resistance of MRSA to 405 nm light killing. In the strain MRSA USA300, we found that 108 J/cm\(^2\) exposure of 405 nm light was only capable of inactivating 0.64 log\(_{10}\) CFU (Figure 2A). However, pre-exposure to 460 nm light (180 J/cm\(^2\)) significantly potentiated the killing of MRSA with 3.58 log\(_{10}\) CFU reduction (*P* = 0.004; Figure 2A). Irradiation with 460 nm alone, at an equivalent dose, was insufficient to elicit any antimicrobial effects. In a clinical strain of MRSA (AF0003), we also observed a significant improvement in the killing efficacy by 405 nm light when 460 nm light was pre-exposed. aBL (405 nm) alone inactivated 1.83 log\(_{10}\) CFU, whereas, the addition of 460 nm light resulted in 3.23 log\(_{10}\) CFU reduction (*P* = 0.02; Figure 2B). As with MRSA USA300, 460 nm irradiation alone, at an equivalent dose, was insufficient to elicit any antimicrobial effects on the strain AF0003.
STX photolysis alone mediated sensitization of MRSA to 405 nm aBL

To determine whether the enhancement in MRSA CFU reduction by 460 nm light was solely based on STX photolysis or whether there is another underlying mechanism, P1::ΔcrtM and its parental WT strain were exposed to 405 nm light or the 460 nm + 460 nm combination. No significant difference in CFU reduction was identified when the S. aureus P1::ΔcrtM mutant was exposed to the dual-wavelength irradiation (460 nm light at 180 J/cm$^2$ and 405 nm at 54 or 108 J/cm$^2$), compared with 405 nm light exposure (54,108 J/cm$^2$) alone (Figure 3A; $P = 0.24$), suggesting that the role of 460 nm light is solely to photolyze STX and subsequently render MRSA more susceptible to ROS generated by 405 nm light. With respect to the WT strain, pre-exposure to 460 nm light (180 J/cm$^2$) significantly enhanced 405 nm aBL killing, further confirming the role of 460 nm light in sensitizing MRSA to 405 nm aBL ($P = 0.001$; Figure 3B).

Enhancement of 405 nm light killing by 460 nm light was dose-dependent

In this study, we determined the effect of increasing radiant exposures of 460 nm light on the effectiveness of 405 nm light. The representative clinical strain of MRSA (AF0003) was used. MRSA was exposed to different radiant exposures of 460 nm light (90 J/cm$^2$, 180 J/cm$^2$, or 360 J/cm$^2$; reflecting a 15, 30 and 60 mins pre-exposure duration, respectively), prior to exposing bacteria to 54 J/cm$^2$ or 108 J/cm$^2$ 405 nm light. Pre-exposure to 360 J/cm$^2$ 460 nm light resulted in the most significant killing of MRSA following 54 J/cm$^2$ 405 nm light, with a killing of 2.14 log$_{10}$ CFU, compared with 405 nm alone which only reduced the CFU by 0.36 log$_{10}$ CFU ($P = 0.002$; Figure 4). Conversely, exposing MRSA to 54 J/cm$^2$ 405 nm light following exposure to 90 J/cm2 or 180 J/cm2 did not result in any significant improvement ($P = 0.9$). This suggests that the enhancement of the antimicrobial efficacy of 405 nm light by 460 nm light is dose-dependent, which is not surprising as increase photolysis of STX by 360 J/cm$^2$ may have increased
sensitivity of MRSA to lower 405 nm doses. When exposure to 405 nm aBL was increased to 108 J/cm², pre-exposure to 180 J/cm² significantly enhanced killing of MRSA relative to 405 nm light alone (P = 0.02). At a radiant exposure of 90 J/cm² 460 nm light, however, there was no significant improvement in the killing when 108 J/cm² 405 nm light was exposed (P = 0.40).

Findings suggest that enhancement of 405 nm light mediated killing by pre-exposure to 460 nm light, is contingent on the delivered radiant exposure of 460 nm light; as increasing radiant exposures of 460 nm light resulted in increased susceptibility of MRSA to 405 nm light. We additionally found there to be some increase in intracellular ROS (1.56-fold) when 405 nm light was administered following photolysis by 460 nm light (360 J/cm²), when compared to 405 nm alone; however, this was not found to be statistically significant (P=0.24; Figure S2)

**Dual-wavelength 460 nm + 405 nm light exposure effectively for reduced the viability of MRSA biofilms.**

In this study, we investigated whether pre-exposure to 460 nm light improved the efficacy of 405 nm aBL against 48-hour old MRSA biofilms. The MRSA AF0003 strain was used as the representative strain for all biofilm experiments. The biofilms were initially exposed to 180 J/cm² 460 nm light, immediately prior to exposing to 405 nm light, at 108, 216 or 324 J/cm². We found that irradiation of 108 J/cm² aBL at 405 nm did not result in any antimicrobial effects in the dual-wavelength irradiation exposed group, or in the 405 nm alone treated group (Figure 5). When the exposure of 405 nm light was increased to 216 J/cm², however, the dual-wavelength irradiation treated group showed a CFU reduction of 1.85 log₁₀, compared with 0.73 log₁₀ CFU reduction in 405 nm light alone treated group (P<0.05). When the exposure of 405 nm light reached 324 J/cm², the dual-wavelength irradiation resulted in 2.72 log₁₀ CFU reduction in MRSA biofilms, compared with 405 nm light alone which inactivated 1.49 log₁₀ CFU (P =0.002; Figure 5).
Dual-wavelength of 460 nm + 405 nm light significantly improved the treatment efficacy in a mouse skin abrasion infection model.

To predict the clinical translatability of the dual-wavelength irradiation approach combining 460 nm and 405 nm light exposure, we investigated its efficacy in a proof-of-principle mouse skin abrasion infection model. Mice were inoculated within an abrasion wound with approximately $10^7$ CFU of MRSA USA 300 and incubated 3 hours prior to treatment with the dual-wavelength irradiation. When the infected wounds were treated with the dual-wavelength irradiation (460 nm/342 J/cm$^2$ and 405 nm/360 J/cm$^2$), a 1.97 log$_{10}$ CFU reduction was achieved, compared with 405 nm alone at an equivalent radiant exposure which only reduced the CFU by 0.28- log$_{10}$ CFU ($P<0.0001$; Figure 6; n=7). Treatment with 460 nm light (342 J/cm$^2$) alone did not influence bacterial viability within the wounds, and 405 nm light alone (360 J/cm$^2$). These findings demonstrate the potential of dual-wavelength (460 nm +405 nm) irradiation approach as a viable treatment option for wound infections caused by MRSA.

Dual-wavelength exposure of 460 nm + 405 nm light did not result in apoptosis of mouse skin cells.

To determine whether the dual-wavelength irradiation by the combination 460 nm and 405 nm light results in cellular apoptosis of mammalian skin cells, the therapeutic dose of the dual-wavelength irradiation used during the in vivo killing studies (460 nm light/ 342 J/cm$^2$ and 405 nm light/360 J/cm$^2$) was administered to naive mouse skin tissue prior to assessing cellular apoptosis using the TUNEL assay. Results showed no presence of apoptotic cells in the treated group immediately following the dual-wavelength irradiation treatment, 24 hours post-treatment, or 48 hours post-treatment (Figure 7A, B, C). The untreated group did not show any evidence of apoptosis (Figure 7D), compared to the positive control (Figure 7E).
In this study, we investigated the role of STX in promoting resistance to 405 nm light killing in MRSA. In addition, we explored the STX photolytic effect of 460 nm light exposure as a means of sensitizing MRSA to 405 nm light killing. With the use of the STX deficient, Pig1::ΔcrtM we confirmed, for the first time, that the presence of STX within MRSA, is directly responsible for the relative resistance phenotype to 405 nm light that has been observed in our and other laboratories\textsuperscript{6}. There have, however, been studies that have illustrated sensitivity of 405 nm light killing, which conflict somewhat to ours\textsuperscript{10}. It is possible that variabilities in the relative abundance of endogenous STX may explain these findings. Furthermore, as STX is present in 90% of strains\textsuperscript{11} it is feasible that improved efficacy of 405 nm light may be achieved using these STX deficient strains, although further work is required to corroborate this hypothesis. Given that STX has a carotenoid structure, and is thus a singlet oxygen scavenger, it is unsurprising that its presence would interfere with damage elicited by singlet oxygen\textsuperscript{12}. Liu et al., fully characterized the role of STX and found it to be a powerful singlet oxygen scavenger. They found the STX deficient isogenic mutant ΔcrtM, was >100-fold more susceptible to singlet oxygen generated through methylene blue mediated photodynamic therapy (MB-PDT), relative to its parental WT\textsuperscript{7}. These findings, therefore, strongly support our findings illustrating the role of STX in promoting resistance to 405 nm mediated killing. In a previous study, it was demonstrated that photolysis using 460 nm light sensitized MRSA to killing by hydrogen peroxide\textsuperscript{9}, further supporting the role of STX in eliciting resistance to ROS resulting from 405 nm light illumination. We next sought to explore whether pre-exposure to 460 nm light would be adequate to overcome the relative resistance observed when MRSA is exposed to 405 nm light. It was demonstrated previously, that 460 nm light illumination was capable of STX lysis in MRSA. We
found that the degree of STX photolysis achieved by 460 nm light illumination significantly potentiated the killing of MRSA when subsequently exposed to 405 nm, suggesting that the extent of STX lysis was enough to overcome any innate resistance to 405 nm light killing. Exposure to 460 nm light alone, however, was insufficient to elicit any antimicrobial effects. Our results are consistent with findings from other studies that found 460 nm light did not significantly influence the viability of MRSA in vitro\textsuperscript{6,9}. In contrast, however, findings from another study demonstrated that at a wavelength of 470 nm, a significant loss of viability may be achieved in \textit{S. aureus}\textsuperscript{13}. Furthermore, a recent study found that combining riboflavin with 450 nm light potentiated killing of MRSA\textsuperscript{14}. Riboflavin has been shown to possess photosensitizing properties, having been activated with UV to eliminate microorganisms\textsuperscript{15,16}. In addition, riboflavin has been found to be effective in combination with 460 nm light for inactivating \textit{Listeria monocytogenes}\textsuperscript{17}, which is not surprising given that riboflavin can absorb light at this wavelength\textsuperscript{18}. Moreover, MRSA can produce riboflavin endogenously, depending on the exogenous availability of riboflavin\textsuperscript{19}. In a study by Chaffin \textit{et al.} 2012, it was found that genes encoding riboflavin biogenesis were not up-regulated when riboflavin was adequately available (such as when grown in Luria-bertani medium\textsuperscript{20}); suggesting that when exogenous riboflavin is limited, MRSA may overproduce riboflavin endogenously. Therefore, it is reasonable to infer that variabilities in the endogenous production of riboflavin may offer an explanation as to the conflicting findings achieved by different studies into the antimicrobial effects of 450-470 nm light.

In addition to antimicrobial killing with the use of light within the blue light spectrum (i.e. 400-470), there have been other light-based modalities that have effectively eliminated organisms. For example, MB-PDT which is a commonly used light-based antimicrobial approach, (which uses a photosensitizer (MB) that is activated by red light to generate ROS) which has been
used against an array of bacterial pathogens, inclusive of MRSA in vitro and in vivo\textsuperscript{21,22}. In a study by Vecchio et al. (2015) they found MB-PDT was able reduce the relative luminescence of a bioluminescent variant of MRSA USA300 inoculated onto a burn (30 minute incubation) by approximately $2\log_{10}\textsuperscript{22}$. This reduction in MRSA was similar to what we observed after 3-hours incubation with MRSA USA300 using the 460 nm + 405 nm light approach. The obvious benefit of our 460 nm + 405 nm light approach is that it is strictly light-based and does not require the addition of any exogenous chemical agent. In addition, other light-based strategies using non-visible light such as Ultraviolet C (UVC; 200-280 nm wavelength) have been employed\textsuperscript{23}. In a study by Dai et al (2012), they investigated the effectiveness of UVC on mouse abrasion wounds containing \textit{S. aureus} or \textit{Pseudomonas aeruginosa}. They found low radiant exposures of UVC (<3 J/cm\textsuperscript{2}) to effectively reduced the bacterial burden of both species\textsuperscript{23}. However, while UVC is an effective approach to eliminate infection, an important consideration are potential side effects on the surrounding mammalian tissue. As UVC can be absorbed by host DNA resulting in DNA damage\textsuperscript{24}; the application of longer wavelengths such as those within the blue light region have been shown to be considerably safer than UVC\textsuperscript{24}.

We next investigated whether STX photolysis by 460 nm light was predominantly responsible for potentiating the effects of 405 nm light against \textit{S. aureus}. We found that in a parental WT strain of \textit{S. aureus} (Pig1), pre-exposure to 460 nm light significantly improved the antimicrobial effects elicited by 405 nm light. However, in an isogenic \textit{ΔcrtM} mutant of the same strain (Pig1) that does not produce STX, similar killing efficacies were observed when exposed to 405 nm light irrespective of 460 nm light pre-exposure. These findings strongly suggest that the improved anti-MRSA efficacy of the dual-wavelength irradiation over 405 nm aBL alone is predominantly due to STX photolysis by 460 nm light, rendering \textit{S. aureus} more susceptible to 405 nm light killing.
As bacterial biofilms are important factors contributing to antimicrobial resistance as well as the re-calcitrant nature of MRSA infections\textsuperscript{25,26}, we investigated whether the dual-wavelength irradiation combining 460 nm and 405 nm light may be a potential modality for eliminating MRSA biofilms. With the exposure of 405 nm light above 108 J/cm\textsuperscript{2}, we found that the dual-wavelength irradiation significantly improved the antimicrobial effects, relative to either light wavelength alone. However, no antimicrobial effects were elicited at \(\leq 108\) J/cm\textsuperscript{2} of 405 nm light, in either the 405 nm alone or 405+460 nm light combination treated group. A possible explanation could be the presence of the extracellular polymeric substance (EPS) produced during biofilm formation, which may have attenuated light delivery resulting in higher radiant exposures being required to elicit antimicrobial effects. Alternatively, MRSA cells within the EPS may be less metabolically active, reflecting a persister cell state that renders them more tolerant to antibiotic stress\textsuperscript{27}, may explain the higher light dose required to elicit antimicrobial effects; further work is required to substantiate this hypothesis. A study by Becker, 2017, demonstrated that light at 453 nm can increase the metabolic activity of keratinocytes, in vitro, although at very low radiant exposure\textsuperscript{28}. Therefore, it is possible that 460 nm light may have increased metabolic activity of persister cells with biofilms, rendering them more susceptible to 405 nm light killing (at 216 J/cm\textsuperscript{2} and 324 J/cm\textsuperscript{2}). Becker, 2017, however, observed a bi-phasic dose response with higher radiant exposures of blue light resulting in a decrease in metabolic activity\textsuperscript{28}. This is not surprising as photo-biomodulation effects are typically found at lower doses than those that would have therapeutic effects\textsuperscript{29}. Therefore, further work evaluating the dosimetry of 460 nm light on MRSA biofilms resulting in increased metabolic activity is warranted, to determine its potential role on persister cell activation.

To predict the clinical therapeutic potential of the dual-wavelength irradiation approach combining 460 nm and 405 nm light, we investigated its efficacy in a proof-of-principle\textit{ in vivo
mouse skin abrasion model of infection. We established that following 342 J/cm² of 460 nm light in combination with 360 J/cm² of 405 nm light was enough to inactivate almost 99% (1.97-log₁₀ CFU reduction) of bacteria within the abrasion wounds of mice. Either wavelength of light on its own, at equivalent doses, was insufficient to result in any significant killing of MRSA, compared with the untreated control. This study, therefore, demonstrated a proof-of-principle approach illustrating that the combination of 460 nm and 405 nm may effectively reduce the MRSA burden within mouse wounds. An important limitation of this study is that using the abrasion infection as a model, coupled with the short incubation of the infection, may not necessarily be reflected in a more established or complicated soft tissue infection. Given then low penetrance of 460 nm and 405 nm light through the skin (0.5 mm)⁶ we would expect that in the absence of more sophisticated light delivery methods, such as with the use of optical clearing agents⁷ or a microneedle array for interstitial delivery⁸, the efficacy of combining 460 nm +405 nm light may be limited.

We additionally found no evidence of apoptosis (immediately following treatment, 24 hours later, or 48 hours later) resulting from combining 460 nm +405 nm light against naïve mouse skin, at a dose required to inactivate approximately 99% of MRSA within a mouse skin abrasion wound; suggesting the therapy may be safely administered. It is important to appreciate, however, that the doses required to significantly inactivate MRSA, within more established wound infections, may necessitate higher therapeutic doses. In addition, the results present only present a qualitative assessment safety denoted by the presence (or absence) of apoptotic cells, thus suggesting that further studies are warranted to quantitatively evaluate the safety of using 460 nm +405 nm light against skin. In conclusion, 460 nm + 405 nm combination therapy may offer an effective and safe approach for the treatment of MRSA wound infections.

**MATERIALS AND METHODS**
Blue Light Sources

Irradiations of 460 nm and 405 nm light were delivered using two light emitting diodes (LED; M405L2 and M470L1; Thorlabs, Newton, New Jersey) with peak emissions of 460 nm and 405 nm, respectively, and a full width at half-maximum (FWHM) of 25 nm. The irradiance was regulated by altering the distance of the light source aperture and the target with the use of a PM100D power/energy meter (Thorlabs, Newton, New Jersey).

Bacterial Strains and Growth Conditions

The bacterial strains used in this study include a clinical strains of methicillin resistant *Staphylococcus aureus* (MRSA) AF0003 strain, that was isolated from an infected soldier deployed in Afghanistan, and IQ00064 that was isolated from an infected soldier deployed in Iraq. In addition, a bioluminescent MRSA USA 300 strain\(^7\), an MRSA Pig1 strain\(^7\). In addition, the isogenic \(\Delta ctm\) mutant from the *S. aureus* Pig1 strain\(^7\) was used for mechanistic studies. With the exception of the *S. aureus* Pig1:: \(\Delta ctm\) strain, all are STX producers (Figure S1). The bacteria were cultured in brain heart infusion (BHI) medium (agar or broth) at 37°C, or in an orbital incubator (37°C; 180 rpm), respectively.

Extraction of STX from *S. aureus* strains. All strains of *S. aureus* used in the study were initially grown overnight on BHI agar from 25% glycerol freezer stocks. Subsequently, bacterial colonies were collected with the use of a 10 μL loop and suspended in 10 mL Phosphate buffered saline (PBS). The CFU was then adjusted to approximately \(10^9\) CFU/mL within PBS prior to extraction of STX. The suspended bacteria were then centrifuged at 4000 x g for 4 minutes to pellet the cells. The supernatant was then discarded and the pellet was suspended in 2 mL of 100% methanol and incubated at 55°C for 3 hours to ensure complete extraction of the pigment.
Following the incubation, the cells were pelleted and the supernatant containing STX was transferred to a 1 mL cuvette. The absorption was then measured using an Evolution 300 UV-Vis Spectrophotometer (ThermoFisher Scientific, USA) in accordance with the manufacturer’s instructions.

**Light Killing (460 nm + 405 nm) of Planktonic MRSA In Vitro**

Bacteria were cultured to mid-log phase and adjusted to approximately $10^8$ CFU/mL in PBS and transferred to a 35 x 12 mm dish. When necessary, bacteria were pre-irradiated with 460 nm light (90-360 J/cm²/100 mW/cm²), prior to 405 nm light (108 J/cm²/60 mW/cm²) exposure. During light irradiation, the bacterial suspension was stirred using a 12-mm magnetic bar (20 rpm) to ensure uniform exposure of cells to aBL. Aliquots (40 μL) of the suspension were then withdrawn at varying time points after the initiation of 405 nm light and the CFU was determined by serial dilution ($10^{-2}$-$10^{-7}$ dilution factors) on BHI agar plates as described previously.

Experiments were performed in triplicate (3 independent replicates spanning 3 different days).

Measuring intracellular ROS following 460 nm + 405 nm light exposure. Bacteria were cultured to mid-log phase and adjusted to approximately $10^9$ CFU/mL in PBS and 200 μL were transferred to a 96 well plate. The suspension was irradiated with 360 J/cm² of 460 nm light, and then 10 μM of CellROX® Green Reagent (ThermoFisher Scientific, USA) was added to the bacterial suspension and incubated for 30 minutes to ensure permeabilization of the probe into cells. The bacterial suspension containing the probe was subsequently irradiated with radiant exposures of 3.6 J/cm², 7.2 J/cm², or 14.4 J/cm² 405 nm light (low radiant exposures selected due to limited photostability of the probe) prior to measuring relative fluorescence emission using a SpectraMax M5 multimode multiplate reader (Molecular devices, USA). The
excitation/emission setting were set to 485/520 nm. Untreated bacteria, 405 nm, and measurements following 460 nm treatment (reflecting the time of 405 nm exposure) were run in parallel. Experiments were performed in triplicate (3 independent replicates spanning 3 different days).

**Light Killing (460 nm + 405 nm) of MRSA Biofilms**

Bacterial suspensions in BHI broth were incubated in 96-well microtiter plates (200 μL/well; approximately $10^6$ CFU/mL) as described previously, for 48 hours to induce biofilm formation.$^{26,27}$ Following incubation, the wells were rinsed three times with 200 μL PBS to ensure removal of residual medium or planktonic cells, while ensuring the adhered biofilms remained intact. Prior to light irradiation, aliquots of 200 μL fresh PBS were added to the wells. The biofilms were pre-irradiated with 180 J/cm$^2$ of 460 nm light at an irradiance of 100 mW/cm$^2$ immediately prior to 405 nm light exposure (60 mW/cm$^2$) until radiant exposures of up to 324 J/cm$^2$ were delivered. The adhered cells within the biofilms were then isolated by thoroughly scraping the appropriate wells with a pipette tip and transferring the 200 μL cell/biofilm suspension to a 1.5 mL microcentrifuge tube. A further 200 μL of PBS were then added to the wells and the biofilm isolation was repeated in triplicate, to ensure adequate removal of bacterial biofilms. The collected cells were subsequently sonicated using a branson 2510 water bath sonicator (Marshall Scientific, LLC), to ensure cells within biofilms became adequately dislodged for subsequent plating and CFU quantification. Experiments were conducted in biological triplicate (3 independent replicates spanning 3 different days).

**Inoculation of MRSA within a Mouse Skin Abrasion Wound**

Mice (Female BALB/c) aged 6–8 weeks and weighing approximately 17–19 g were procured from Charles River Laboratories (Wilmington, MA). All animal procedures were approved by the
Institutional Animal Care and Use Committees of Massachusetts General Hospital (protocol number: 2015N000187) in accordance with National Institute of Health guidelines. Prior to producing the skin abrasion wounds in mice, mice were injected intraperitoneally with the use of a ketamine/xylazine cocktail (20 mg/kg -100 mg/kg). The mice were then shaved, and the tissue was carefully abraded within a defined 1.0-cm x 1.0-cm area using a #15 sterile scalpel blade. The scraped area either did not produce any blood or bled very little. Immediately following the abrasion, a 100 µL bacterial suspension containing approximately $10^7$ CFU of MRSA USA300 in PBS was inoculated onto the wound and applied gently and uniformly using the side of a pipette tip and left to incubate for 3 hours prior to treatment. To ensure that the inoculum was left intact within the mouse abrasion wound, mice were kept anaesthetized until the inoculum dried within the wound. In addition, to limit physical removal of bacteria from the wound, bacteria were isolated within cages, with no more than 2 mice/cage. Experiments were performed in septuplicate (7 independent replicates spanning 7 different days).

**Light Killing (460 nm+405 nm) of MRSA within mouse skin abrasion wounds**

Three hours following inoculation of mouse abrasion wounds, MRSA was treated within mouse wounds. During light exposure *in vivo*, an adjustable collimator was attached to each light source, 460 nm and 405 nm light, and the LEDs were held 16 cm from the target, angled at an approximately 45°, and the collimated light beam was adjusted cover the abrasion wound (see Figure 8). The 460 nm light was initially delivered onto the infected wound for 15 mins (200 mW/cm²), prior to irradiation with 405 nm light (60 mW/cm²). Once the 405 nm light was initiated, the 460 nm light irradiance was lowered to 30 mW/cm² and exposed continuously during 405 nm light illumination (90-minute duration). The purpose of continuous exposure of
460 nm light was to ensure continuous photolysis occurred during 405 nm illumination, as it was found that in a medium that supports MRSA growth (i.e. *in vivo*) STX can become replenished when 460 nm light exposure ceases. The total radiant exposure of 460 nm light was 342 J/cm² and 405 nm light was 360 J/cm² (reflecting a 105-minute treatment time). A total of 7 mice were included for each group and experiments were performed over 7 different days (1 mouse for each group per day).

**Quantification of bacterial CFU in mouse wounds following light treatment**

Immediately following treatment, mice were euthanized in accordance with standard procedures, and the 1-cm x 1-cm infected skin tissue was isolated and homogenized in 1 mL PBS within a FastPrep lysing matrix tube A (MP Biomedicals, USA) in a FastPrep-24™ Classic Instrument (MP Biomedicals, USA). Samples were homogenized for 5 minutes, with 60 s intervals, and samples were placed on ice for 5 minutes in between homogenization cycles, to limit heat generation. Following homogenization, the CFU was determined by serial dilution (10⁻²⁻¹₀⁻⁷ dilution factors) on BHI agar plates as described previously.

**TUNEL assay to detect apoptotic cells in mouse skin treated with 460 nm + 405 nm light**

The presence of apoptotic cells that resulted from the dual-wavelength irradiation therapy was determined in healthy mouse skin as described previously. In brief, skin from the mouse was isolated at 0 and 24 hours, following the treatment with 460 nm light (342 J/cm²) and 405 nm (360 J/cm²). An untreated skin sample was also included as the control and was immediately fixed in a 10% phosphate-buffered formalin (Fisher Scientific) for 48 hours. Tissue sections (4 μm thick) were then analyzed with the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) in accordance with the manufacturer’s instructions. Fluorescence images were visualized with the use of NanoZoomer S60 Digital slide scanner where a fluorescein
isothiocyanate was used as the fluor, and DAPI as the nuclear counterstain. In addition, a
DNase I (RQ1 RNasefree DNase, Promega, USA) treated section (which induces significant
DNA damage) served as a positive control mouse skin. Fluorescence images were visualized
with the use of NanoZoomer S60 Digital slide scanner where a fluorescein isothiocyanate was
used as the fluor, and DAPI as the nuclear counterstain. In addition, a DNase I (RQ1 RNasefree
DNase, Promega, USA) treated section (which induces significant DNA damage) served as a
positive control.

Statistical analyses

Data were presented as the mean ± standard error (SE), with differences between means being
compared for significance, where appropriate, by either a paired t-test or a one-way analysis of
variance (ANOVA). P-values of <0.05 were considered significant.

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mutant.

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CONFLICTS OF INTEREST
CONTRIBUTIONS

T.D. proposed concept of dual-wavelength photo-killing of MRSA. L.G.L and XG established a proof-of-principle for the therapeutic modality. L.G.L performed in vitro and in vivo studies. JXC and DCH helped provide mechanistic insights into the dual-wavelength photo-killing approach, aiding in the study design. T.D. and L.G.L designed experiments and wrote the paper.

REFERENCES


Figure 1: A) killing kinetics of *S. aureus* Pig1 WT and the Δ*crtM* mutant by 54 J/cm$^2$ and 108 J/cm$^2$ 405 nm light, or 460 nm light (180 J/cm$^2$). Error bars: standard error of the mean ($n=3$). ***=$P \leq 0.001$ (paired t-test: comparing 405 nm (WT) vs. 405 nm (Δ*crtM*) mutant).
Figure 2: A) killing kinetics of MRSA USA300 B) MRSA AF0003 and C) MRSA IQ00064 by 405 nm light (54 J/cm² or 108 J/cm²), 460 nm + 460 nm light (pre-exposure to 180 J/cm² 460 nm light followed by 54 J/cm² or 108 J/cm² 405 nm light) or 460 nm light (180 J/cm²). Error bars: standard error of the mean (n=3). *=P≤0.05, **=P≤0.01 (paired t-test – comparing 405 nm vs. 460 nm + 460 nm).
Figure 3: A) of *S. aureus* Pig1::ΔcrtM mutant and B) *S. aureus* Pig1 (WT strain) with 405 nm, 460 nm + 460 nm or 460 nm. Error bars: standard error of the mean (n=3). ***=P≤0.001 (paired t-test comparing 405 nm vs. 460 nm + 460 nm).
Figure 4: killing kinetics of MRSA by 405 nm light, following radiant-exposures of 54 J/cm$^2$ or 108 J/cm$^2$, with or without pre-exposure to 90 J/cm$^2$, 180 J/cm$^2$ or 360 J/cm$^2$. Error bars: standard error of the mean ($n=3$). *$=P \leq 0.05$, **$=P \leq 0.01$ (paired t-test comparing 405 nm vs. 460 nm + 460 nm + 460 nm).
Figure 5: A) Killing kinetics of MRSA AF0003 48-hour biofilms, following exposure to 405 nm light (108 J/cm², 216 J/cm² or 324 J/cm²), 460 nm + 460 nm light (pre-exposure to 180 J/cm² 460 nm light followed by 108 J/cm², 216 J/cm² and 324 J/cm²) or 460 nm light (180 J/cm²). Error bars: standard error of the mean (n=3). *P≤0.05, ***P≤0.001 (paired t-test comparing 405 nm vs. 460 nm + 460 nm).
Figure 6: Scatter plot showing log_{10} CFU/cm^2 following: no treatment (control), 460 nm light treatment, 405 nm light treatment and 460 nm + 405 nm light treatment. Each point represents an individual value with the mean value being represented by the horizontal line. Error bars: standard error of the mean (n=7). The statistical methods employed were a paired t-test comparing 2 individual conditions (405 nm vs. 460 nm + 405 nm; 405 nm vs. 460 nm) and a one-way ANOVA compared 3 or more groups (highlighted by the horizontal lines spanning the different groups).
Figure 7: Representative images showing TUNEL stained mouse skin sections for the detection of apoptotic cells resulting from: A) 0 hours post-treatment with 460 nm light (342 J/cm²) + 405 nm light (360 J/cm²) B) 24 hours post-treatment with 460 nm light (342 J/cm²) + 405 nm light (360 J/cm²). C) 48 hours post-treatment with 460 nm light (342 J/cm²) + 405 nm light (360 J/cm²). D) no treatment. E) a positive control treated with DNase I was also added. Florescence of fluorescein and DAPI are represented by green (indicative of fluorescein binding to damaged DNA) and blue (DAPI stain of intact nuclei) pseudo-color, respectively. DAPI is a nuclear counterstain. White circle indicates apoptotic cell – indicated by green fluorescence. Bar: 250 μm.
Figure 8: Experimental set-up for the treatment of MRSA within mouse wounds.
Figure S1: Representative images of STX extract from A) MRSA Pig1 and B) MRSA Pig1: ΔcrtM. C) STX absorbance spectra from the extracts of all the MRSA strains used in the study (Pig1, Pig1: ΔcrtM, USA300, AF0003, and IQ00064.)
Figure S2: A) kinetics of ROS production (indicated by the RFU) with increasing radiant exposures of 405 nm light (3.6 J/cm², 7.2 J/cm², and 14.4 J/cm²), 460 nm +405 nm light (pre-exposure to 360 J/cm² 460, followed by 3.6 J/cm², 7.2 J/cm², and 14.4 J/cm² 405 nm light), control (untreated bacteria), and 460 control (ROS production immediately following 460 nm illumination (360 J/cm²)). B) AUC analysis illustrating the total RFU achieved with increasing radiant exposures of 405 nm light (3.6 J/cm², 7.2 J/cm², and 14.4 J/cm²), 460 nm +405 nm light (pre-exposure to 360 J/cm² 460, followed by 3.6 J/cm², 7.2 J/cm², and 14.4 J/cm² 405 nm light), control (untreated bacteria), and 460 control (ROS production immediately following 460 nm illumination (360 J/cm²)). Error bars: Standard error of the Mean (n=3). NS = not significant (paired t-test comparing 405 nm vs. 460 nm + 460 nm).