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# Harnessing the Power of Light to Treat Staphylococcal Infections Focusing on MRSA

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**Abstract:** Methicillin-resistant *Staphylococcus aureus* (MRSA) has become the most important drug-resistant microbial pathogen in countries throughout the world. Morbidity and mortality due to MRSA infections continue to increase despite efforts to improve infection control measures and to develop new antibiotics. Therefore alternative antimicrobial strategies that do not give rise to development of resistance are urgently required. A group of therapeutic interventions has been developed in the field of photomedicine with the common theme that they rely on electromagnetic radiation with wavelengths between 200 and 1000 nm broadly called "light". These techniques all use simple absorption of photons by specific chromophores to deliver the killing blow to microbial cells while leaving the surrounding host mammalian cells relatively unharmed. Photodynamic inactivation uses dyes called photosensitizers (PS) that bind specifically to MRSA cells and not host cells, and generate reactive oxygen species and singlet oxygen upon illumination. Sophisticated molecular strategies to target the PS to MRSA cells have been designed. Ultraviolet C radiation can damage microbial DNA without unduly harming host DNA. Blue light can excite endogenous porphyrins and flavins in MRSA cells that are not present in host cells. Near-infrared lasers can interfere with microbial membrane potentials without raising the temperature of the tissue. Taken together these innovative approaches towards harnessing the power of light suggest that the ongoing threat of MRSA may eventually be defeated.

**Keywords:** ??????????????????????.

## INTRODUCTION

The introduction of penicillin was hailed as the dawn of a new age in medicine. No longer would relatively minor injuries and infections lead to death from uncontrolled sepsis. Little did the medical profession realize, but the new"wonder-drug" contained the seeds of its own destruction. Within a few years of its introduction in 1946, reports of resistance to penicillin in Staphylococci began to emerge [1]. While we should not underestimate the health benefits of antibiotics to mankind in general, or fail to acknowledge the number of lives saved throughout the world by judicious use of antibiotics, it is becoming accepted that we are now at the "end of the antibiotic era" [2]. The lack of new drugs in the pipelines of big pharmaceutical companies combined with reports of ever widening spread of microbial resistance has driven the search for new antimicrobial approaches, against which it is hoped, microbes will be unable to develop resistance.

Fortunately this search has proved fruitful [3]. An ever-broadening array of ingenious antimicrobial technologies has emerged that have been termed "disruptive innovations" [4]. This article is concerned with the use of light-based antimicrobial techniques [5]. By its very nature a light-based approach is more suited to tackle localized infections, rather than disseminated systemic infections. Nevertheless, this consideration does not detract from the usefulness of light as an antimicrobial as most human infections (especially bacterial and fungal) start by relatively confined colonization of tissues by microbial cells. If the protective barrier of the tissue is disrupted by injury, or if the host defense mechanism is weakened by metabolic disturbance, by comorbid conditions or immune suppression, these initial minor microbial colonizations can spread and expand into full-blown infectious disease. These considerations are particularly applicable to infections by *Staphylococcus aureus* and its methicillin-resistant strains known as MRSA. The relatively ubiquitous nature of MRSA organisms in the present-day environment (both in the hospital and the community) means that they can easily contaminate wounds (both traumatic and surgical). These localized infections are ideally suited to be treated by light-based techniques, where the chief obstacle to effectiveness is whether the wavelength of light chosen can sufficiently penetrate the tissue to enable efficient bacterial destruction without causing unacceptable harm to the host tissue.

The wavelengths covered in the present review range from the UV to the IR (200-1000 nm), and this sequence roughly parallels the ability of light to penetrate tissue (Fig. **1**). The most investigated light-based antimicrobial approach is that of photodynamic inactivation (PDI) where the light is combined with a photoactivatable dye called a photosensitizer (PS), but several different approaches use light alone to kill pathogens.

## EPIDEMIOLOGY OF MRSA: VINCENT

MRSA remains one of the most prevalent multidrug-resistant organisms causing health care-associated infections [6]. Globally, it is a common cause of nosocomial and community-acquired infections and remains an important public health problem [7, 8]. Within the United States, more than 80, 000 invasive MRSA infections were reported in 2010 [9] , but the annual incidence varies across states [10, 11] substantially impacting health care costs. Although MRSA is generally endemic, an outbreak was reported in a neonatal unit with a rare highly virulent community-acquired pathogen rapidly spreading through nosocomial transmission [12]. The majority of patients reporting to hospitals with MRSA colonization or infec-

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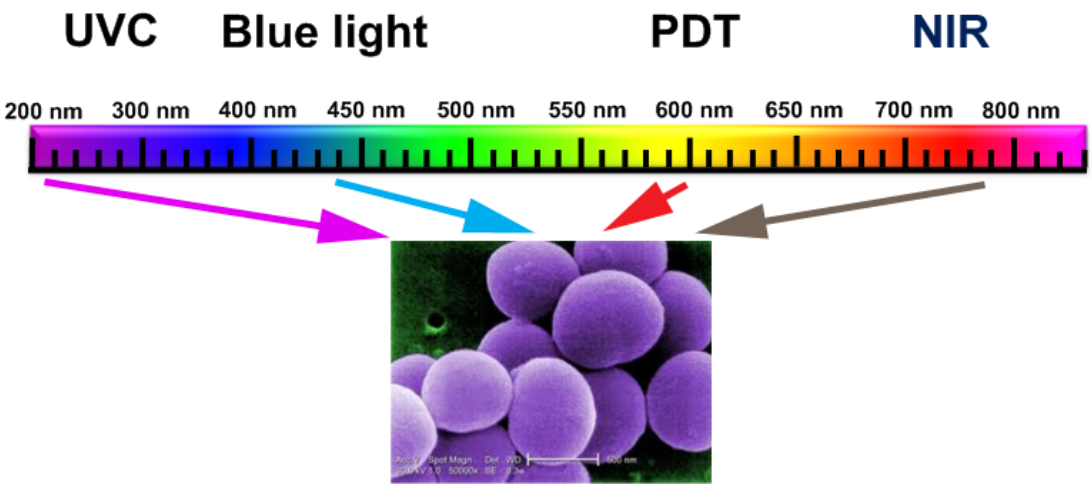


**Fig. (1).** Electromagnetic spectrum contains a set of different colors (wavelengths) of light. These wavelengths can be used to kill MRSA via different mechanisms (alone or in combination with a photosensitizer).

tion are diagnosed within 48 hours of admission, with the most common site of infection being the skin and soft tissue [13]. MRSA colonization is a significant predictor for infection; but there are contradicting reports regarding age as a risk factor [14, 15]. Generally, using the Centers for Disease Control and Prevention's definitions, more than 50% of MRSA cases are classified as health care-associated infections [16]. Over the years, the rate of MRSA infection has decreased while that of colonization has increased [13], with the community-associated MRSA accounting for a growing proportion of hospital-onset infections [17]. Surveillance and prevention of MRSA are therefore a priority in current infection control programs. The economic impact of community-associated methicillin-resistant Staphylococcus aureus is enormous. A single CA-MRSA case can cost up to $20 500 and the annual burden may be as high as $13.8 billion. The US jail system and Army may be experiencing annual total costs of $11 million and $36 million respectively [18].

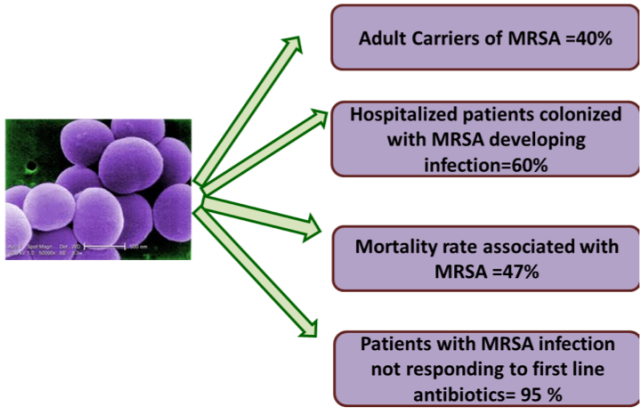


**Fig. (2).** Epidemiology of MRSA- MRSA is most common community-acquired infection and is a major public health problem

## RESISTANCE MECHANISMS

Staphylococcal infections have been associated with key multidrug resistance phenotypes due to the possession or acquisition of unique antibiotic resistance and virulence traits that are generally attributed to a variety of mobile genetic elements (MGE) as well as chromosomal determinants. Among MGE the prophages that carry the species-specific Panton-Valentine leukocidin (PVL) toxin have been viewed as the cornerstone of resistance for of CA-SA strains [19, 20]. The PVL-carrying prophages are temperate bacteriophages that belong to the ds-DNA (double stranded) phages family of *Siphoviridae. They* carry a long non-contractile tail and the capsid morphology is the basic criterion for the identification of 3 distinct groups: group 1 (φPVL, φ108PVL), group 2 (φSa2958, φSa2MW, φSLT, φSa2USA) or group 3 (φ7247, φ5967, φM013). The PVL toxin contains two hetero-oligomeric components LukF-PV (34 KDa) and LukS-PV (33 KDa) [21]. PVL lyses human white blood cells by forming pores and shows specific interactions with components of the immune system [22]. The exact role of the PVL-toxin in virulence and pathogenicity is controversial and requires thorough investigation. A study from Yoong and Pier [23] implies that at sublytic concentrations PVL enhances innate host immunity without causing substantial cell damage. The results from rabbit infection models suggests that PVL has a contributing but not the major role in CA-MRSA infections [24]. A recent study further demonstrated that PVL is the major trigger for IL-1β release and inflammasome activation in human macrophages [25]. An array of new preclinical studies suggest a species-specific role for PVL in the development of acute severe *S. aureus* infections [26]. PLV seems to contribute to pathogen invasion and internalization, driving the regulation of virulence factors and defining the major pathogen post-invasion strategies and phenotypic switching.

Two key chromosomal loci have been intensively investigated 1) the Staphylococcal Cassette Chromosome *mec (SCCmec),* which carries determinants of antibiotic resistance [27, 28] *SCCmec* is the genomic island with species specific nucleotide sequences encoding for methicillin resistance. Methicillin-susceptible *S. aureus* (MSSA) changes to MRSA upon the acquisition of *SCCmec*, 2) the accessory gene regulator *agr* system [29] a global chromosomal regulator of virulence with a substantial role in quorum sensing, and biofilm formation. Chung *et al* [30] demonstrated the critical role of *agr* fin CA-MRSA infections with a variety of USA300 phenotypes. Regulation of *agr* leads to exceptionally strong expression of toxins and exoenzymes, upregulation of fibrinogen-binding proteins, and increased expression of methicillin resistance genes.

There are also key resistance issues emerging in MSSA as well as MRSA infections with the most prominent being: 1) Extensive reports of elevated vancomycin MICs for MSSA attributed to a variety of resistance mechanisms [31]; 2) linezolid resistant clonal isolates that have acquired the chloramphenicol/florfenicol resistance gene [32]; and 3) case reports of clinical failure attributed to

daptomycin resistance with a mechanism that remains unclear at this stage [33].

## PHOTODYNAMIC INACTIVATION FOR MRSA INFECTIONS

Photodynamic therapy (PDT) is a technique to kill cells and destroy tissue that employs photosensitizers (PS) activated by light of appropriate wavelength [34-36]. The initial excited singlet state of the PS undergoes intersystem crossing to form the long-lived excited triplet state of the PS that acts as a reactive intermediate to generate reactive oxygen species (ROS) (Fig. **3**). When the PDT approach is used as an anti-infective or antimicrobial it is usually termed photodynamic inactivation (PDI) [37-39]. The ROS initiate further oxidative reactions locally with components of the staphylococcal bacterial cell wall, cell membranes, enzymes or nucleic acids [40]. However, one of the challenges in employing PDT with MRSA is to look for appropriate PS suitable to inactivate the MRSA without affecting the neighboring host cells. Some of the chemical structures of PS that have been used to inactivate MRSA and MSSA are depicted in Fig. **4**.

Favorable results were achieved in inhibition of methicillin-resistant *S. aureus (*MRSA) *in vitro* with antimicrobial photodynamic therapy. A 15-minute exposure to a 632.8 nm HeNe laser in the presence of 50 µg/mL photosensitizer toluidine blue O (TBO) (Fig. **4A**) completely eradicated MRSA.[41].

In addition, a light-activated antimicrobial agent aluminum disulfonated phthalocyanine (AlPcS2) (Fig. **4B**) has shown that 16 epidemic MRSA strains (EMRSA) could be inactivated by antimicrobial PDT. [42]. The AlPcS2 concentration and the light dose were responsible for the bactericidal effect and was not affected by the growth phase of the organism. The scavengers of singlet oxygen and free radicals provided protection from the bactericidal effect [42].

A deep understanding of the effect of photosensitizers on MSSA vs MRSA comes from studies by Grinholc group [43-46]. In a recent study, they aimed to investigate any significant correlation between bacterial toleration of oxidative stress, porphyrin level, photosensitizer uptake and strain's virulence between MSSA and MRSA strains and their response to photodynamic inactivation (using protoporphyrin diarginate (PPArg2)) (Fig. **4C**), TBO and 5-aminolevulinic acid (ALA) (Fig. **4D**). The study revealed that that PDI by PS namely PPArg2, ALA and TBO would eliminate the S.aureus elevated resistance in both highly virulent, low virulent and extracellular fraction of S. aureus strains. However, they could not explore the underlying mechanism of strain-dependent response to photoinactivation [43]. They also demonstrated that depending on the PS used the same bacterial isolate could be categorized as highly resistant or highly sensitive to PDI. Additionally, the same PS could totally eradicate some bacterial strains and could be non-effective in the case of other strains. Furthermore, they were able to reverse the resistant to PDI by a change in the PS. Therefore, in order to make a PS reliable several sensitizing agents and several isolates of the same bacterial species should be considered [46].

The same group determined that the genetic factor associated with high pathogenicity of S. aureus strains is the agr locus, which translates a molecule accountable for stimulation of virulence genes. The MRSA possesses a gene which is a part of a mobile genetic element known as Staphylococcal Chromosome Cassette mec (SCCmec). Polymorphic differences in the agr locus and SCCmec cassette enable classification of strains into different groups. The strains were incubated with PS PPArg2 for 18 hours and were irradiated with a red light at a dose of 12 ,ÄâJ/cm(2).The studies confirmed that the response to PDI varies among different S. aureus strains. Unfortunately, they could not determine a diagnostically significant pattern, which could assist in the estimation of strain specific response to PDI [45]. The same group examined the correlations between the functionality and polymorphisms of agr

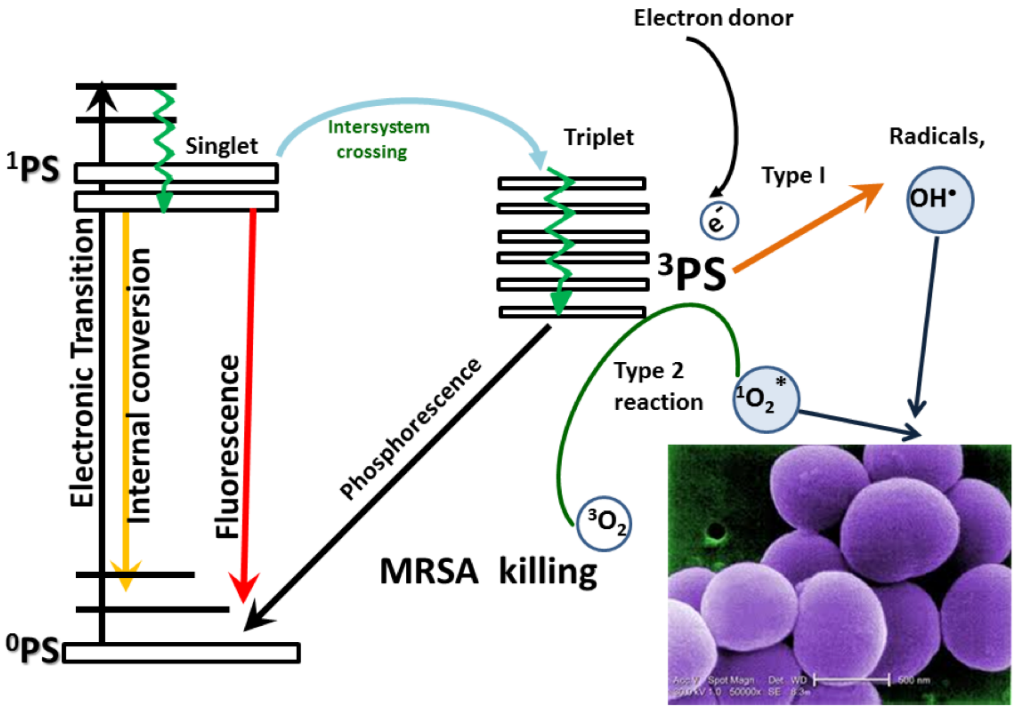


**Fig. (3).** Jablonski energy diagram showing the mechanisms of PDT: The initial excited singlet state of the PS undergoes intersystem crossing to form the long-lived PS triplet state. This triplet state can undergo two parallel pathways. Type I photochemistry involving electron transfer reactions can produce hydroxyl radicals. Type II photochemistry involving energy transfer can produce singlet oxygen. Both these reactive oxygen species are cytotoxic to bacterial cells.

gene determined for 750 MSSA and MRSA strains and their responses to PDI using protoporphyrin IX (Fig. **4E**). The study revealed, the functionality of the agr system affected S. aureus susceptibility to PDI. They concluded that the agr gene may be a genetic factor affecting the strain dependent response to PDI [44].

Tang et al. were the first to report that PDI-mediated killing was more sensitive to isolated MRSA in comparison to S. aureus (ATCC 25923), using two cationic PSs namely, poly-L-lysine chlorin(e6) conjugate (pL-ce6) (Fig. **4F**). and TBO [47].

In recent years the 'nanotechnology revolution" has affected many antimicrobial technologies [48, 49] and antimicrobial PDI is no exception [50]. Nanotechnology approaches have been used to carry out PDT of *S. aureus* and of MRSA. A 6 $\log_{10}$ reduction was observed for MRSA by using a synthetic preparation of porphyrin-cellulose-nanocrystals (CNC-Por(1)) [51]. In this experiment a 20 μM suspension of CNC-Por(1) was treated in combination with visible light (400–700 nm) at a fluence of 118 J/cm$^2$. Silica nanoparticles decorated with Rose Bengal showed improved efficacy of PDT against MRSA *in vitro* and was suggested to be tested *in vivo* [52]. A lipid-bilayer vesicle delivery vehicle, a type of liposomes, was shown to enhance the delivery of PS to bacteria *in vitro* and would be a candidate for delivery of PS to MRSA *in vivo* [53].

PDT is almost unique amongst antimicrobial technologies in that it is not only able to effectively kill the microbial cells but the ROS generated can also inactivate secreted virulence factors that are proteins, enzymes or lipids [54]. A combination of MB with laser light of 665nm( dose1.93J/cm2) inhibited the activities of V8 protease, alpha-haemolysin and sphingomyelinase expressed by epidemic MRSA16 in a dose-dependent manner (1–20 μM), indicating that PDT reduces the detrimental effect of preformed virulence factors on the host [55]. In another study, *in vitro* incubation of MRSA with different concentrations of TMPyP (5, 10, 15 20-tetrakis(1-methylpyridinium-4-yl)-porphyrin tetra p –toluenesulfonate) (Fig. **4G**) for 10 s and illumination with visible light (50 mW cm(-2)) for 10 and 60 s resulted in a photodynamic killing of ≥99.9% of MRSA at a concentration of 1 μmol l(-1) of TMPyP and an applied radiant exposure of 0.5 J cm(-2) . Incubation with higher concentrations (up to 100 μmol l(-1)) of TMPyP caused bacteria killing of >5 log(10) (≥99.999%) after illumination [56].

The studies covered above have relied on classical bacterial targeting usually involving cationic charged PS that preferentially bind to bacteria. However different strategies have been developed that use specific biochemical recognition to target the PS to MRSA.

Antibody conjugated with several photosensitizers was described as a very hopeful directing PDT [54]. Wilson's group hypothesized that Immunoglobulin G-tin(IV)chlorine e6 conjugate (Fig. **4H**) as a lethal photosensitizer of MRSA [57]. Protein A is expressed and localized in the cell wall protein of few MRSA strains which binds to various isotypes of immunoglobulin G through the Fc region to protein A [57]. In another study by the same group the strains of EMRSA-16 and Streptococcus sanguis were exposed to light from a helium/neon laser in the presence of an IgG–SnCe6 (Fig. **4I**) conjugate. Suspensions were irradiated in the presence or absence of the conjugate (and unconjugated PS) and suspensions kept in the dark in the presence of the conjugate. EMRSA-16 was killed by IgG–SnCe6 and SnCe6 in a light-dose- and PS dependent manner though higher kill rates were achieved with the IgG–SnCe6 than with the unconjugated SnCe6. It was also found that the IgG–SnCe6 conjugate was able to kill EMRSA-16 selectively via protein A. The clinically important EMRSA-16 strain was the most susceptible. Exploring further [58] the same group used an Ab-SnCe6 conjugate to kill EMRSA-16 selectively in a mixed suspension of EMRSA-16 and *Escherichia coli.* Compared with the coagulase-negative staphylococcus species, *S. epidermidis*, EMRSA-16 showed higher killing. Even though there are many encouraging studies, antibody targeting antibacterial therapy has shown a little success in both PDT or cancer therapy. The difficulties associated with antibody-based photodynamic therapies are difficulty to achieve specific antibodies that exhibit high affinity, inconsistent expression of target antigens, and difficulty to internalize antibodies by the same cells [59].

Beta-lactam is resistance is one of the major strategies drug-resistant bacteria have evolved, to achieve enhanced target specificity with limited host damage [60]. A recent study by Zheng et al [60] reported a novel antimicrobial PDT strategy that exploits this resistance mechanism. This novel strategy comprises a prodrug construct with a PS and a quencher linked by beta-lactam ring, resulting in reduced phototoxicity. This unique construct, beta-lactamase enzyme-activated-photosensitizer (beta-LEAP), requires the presence of both bacteria and light for activation and remains inactive otherwise. The beta-LEAP construct consisted of a cephalosporin core structure at the center that provided a cleavable β-lactam ring. The opposite sides of cephalosporin have covalently attached methylene blue as the PS and a quencher. The close approximation of PS and quencher (~3nm) assures that instead of generating the active molecular ROS, the energy absorbed by PS is efficiently transferred to the quencher. Thus, photosensitivity of beta-LEAP is lower compared to free PS. When applied to bacteria expressing the β-lactamase enzyme the beta-LEAP construct is recognized by the active site as a regular cephalosporin antibiotic, which initiates β-lactamase hydrolysis and releases the active PS. Interestingly, beta-LEAP demonstrated specificity towards cleavage both by purified beta-lactamase enzyme and by beta-lactamase over-expressing MRSA cells. Although dark toxicity was comparable to the MSSA reference strain, specific PDI toxicity was detected against MRSA. The study anticipated that when used in combination of standard antibiotic therapy it will destroy both resistant strains by PDI as well as non-resistant bacteria by antibiotics [60].

Exploring further PS Maisch et. al. used an ex vivo porcine skin model to examine the penetration as well as antibacterial efficacy of porphyrin-based PS XF73 (Fig. **4J**) against MRSA. Different concentrations (0-10 microM) and different incubation times (5-60 min) were used to determine phototoxicity against both MRSA and MSSA. A growth reduction of up to 4 logs independently of the antibiotic resistance pattern of used S.aureus strains was observed during antibacterial PDI. The study proved that XF73 PS had concentration-dependent differences in killing efficacy of MRSA in comparison to skin cells using an ex vivo porcine skin model. Clinically, topical delivery of XF73 might be a possible treatment in patients with superficial infections of the skin [61].

A study examined the effect of PDT on bacterial inactivation and wound healing using bioluminescent–MRSA in a mouse skin abrasion model. Tetracationic Zn(II)phthalocyanine (RLP068/Cl) derivative (Fig. **4K**) and TBO were used for the experiment. *In vivo* real-time bioluminescence imaging and wound healing using digital photography was carried out to investigate the light-dose response of PDT and the post-treatment recurrence The study revealed that PDT with RLP068/Cl markedly killed the bacteria and inhibited bacterial re-growth after the treatment, however, this effect was not observed in the case of TBO [62].

A mouse model of skin abrasion wound infected with bioluminescent-MRSA strain (a derivative of ATCC 33591) was developed by Dai *et al.* [63]. PDT was performed using a combination of polyethylenimine (PEI)-ce6 PS (Fig. **4L**) and non-coherent red light. The of bacterial luminescence from the mouse wound was reduced by the PDT in a dose dependent manner until over 99% was gone at 240 J/cm2 compared to wound infected with MRSA and treated with PEI–ce6 only (dark control) where luminescence was largely preserved (Fig. **5A**). PDT induced on average 2.7 log(10) reduction of bacterial luminescence in a light dose dependent manner after 360 J/cm2 light was delivered, (Fig. **5B**).The time courses of the mean bacterial luminescence intensity of the

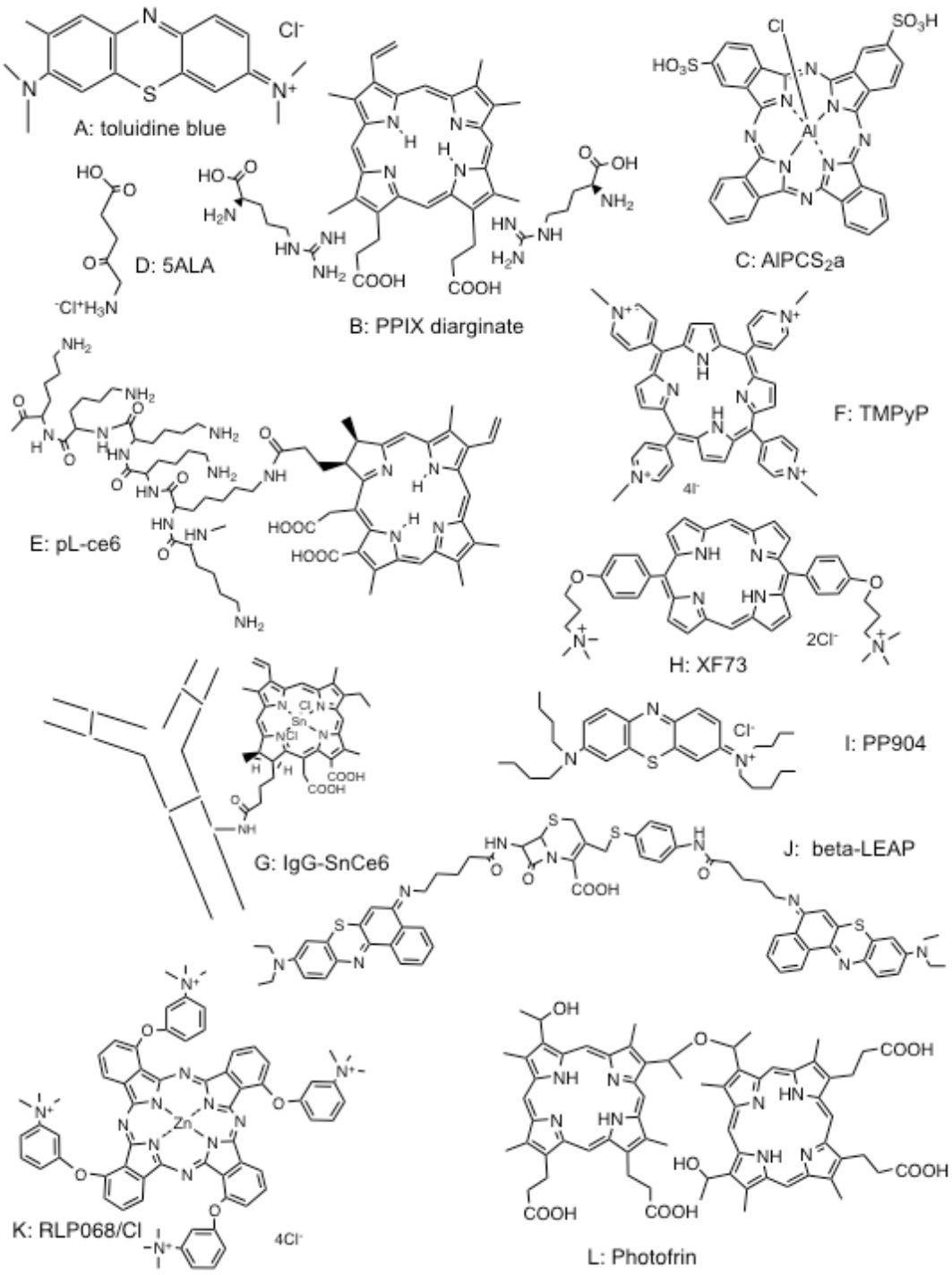


**Fig. (4).** List of some of the PS compounds discussed in the manuscript.

a. toluidine blue (TBO)
b. protoporphyrin diarginate (PPIX-diarg)
c. aluminum disulfonated phthalocyanine (AlPcS2)
d. 5-aminolevulinic acid (ALA)
e. poly-L-lysine chlorin(e6) conjugate (pL-ce6)
f. (Figure-4F) α,β,γ,δ-Tetrakis(1-MethylpyridiniuM-4-yl)porphyrin p-Toluenesulfonate (TMPyP)
g. Immunoglobulin G-tin(IV)chlorin(e6) conjugate (IgG-SnCe6)
h. 5,15-bis-[4-(3-Trimethylammonio-propyloxy)-phenyl]-porphyrin (XF73)
i. (Figure-4I) 3,7-bis(N,N-dibutylamino) phenothiazin-5-ium bromide (PPA904)
j. β-lactamase-enzyme-activated photosensitizer (β -LEAP)
k. Tetracationic Zn(II)phthalocyanine (RLP068/Cl)
l. Photofrin1

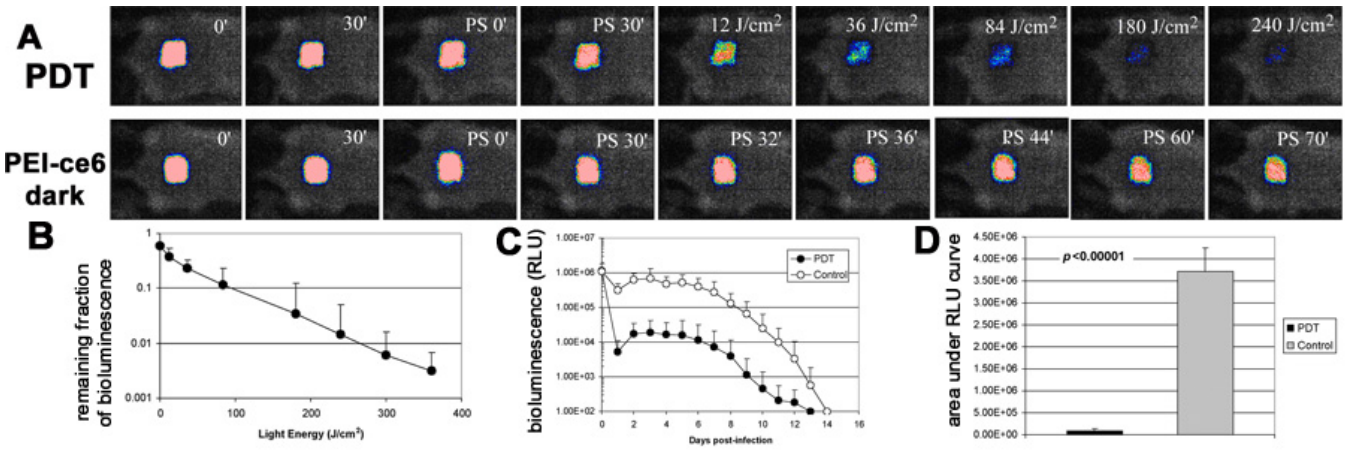


**Fig. (5). A**: Dose–response of bacterial luminescence of a mouse abrasion wound infected with MRSA and treated with PDT; and dose–response of bacterial luminescence of a mouse abrasion wound infected with MRSA and treated with conjugate PEI–ce6 only. PDT was carried out at 30 minutes after infection**. B**: Dose–response of mean bacterial luminescence of the mouse wounds infected with MRSA and treated with PDT(n=10). **C:** Time courses of bacterial luminescence of the infected abrasion wounds in the PDT-treated mice (n=10) and non-treated mice (n=12). **D:** Mean areas under the bioluminescence versus time plots (in the two-dimensional coordinate system in (C), representing the overall bacterial burden of mouse abrasion wounds in different groups. Bars: standard deviation.

PDT-treated wounds after re-growth was on average 1.3-log10 lower than that of the non-treated wounds at the same time points (Fig. **5C**). PDT significantly decreased bacterial bio-burden of the infected wounds as shown by statistical comparison of area under curve of Fig C ($P<0.0001$) (Fig. **5D**) [63].

Tanaka et al studied the *in vitro* effect of PDT on murine peripheral-blood neutrophils using different PS at a concentration that exerted a maximum bactericidal effect on MRSA. The study revealed that PDT using TB or MB can preserve host neutrophils and exert a therapeutic effect on *in vivo* local microbial infection [64]. The same group studied, a murine MRSA arthritis model using bioluminescent MRSA and polystyrene microparticles using MB as PS. They observed that, neutrophils were accumulated into the infectious joint space after PDT and MRSA arthritis was reduced. In preventive PDT regimen bacterial growth was suppressed and the establishment of infection was inhibited. : The study was first to demonstrate a defensive innate immune response against MRSA by PDT [65].

In a mouse model of MRSA arthritis PDT mediated by intra-articular injection of Photofrin1 (Fig. **4M**) and 635-nm diode laser illumination with a range of fluences within 5 minute the greatest reduction of MRSA CFU seen with a fluence of 20 J cm2. A significantly higher concentration of macrophage inflammatory protein-2, a CXC chemokine, and greater accumulation of neutrophils were seen in the infected knee joint after PDT with a fluence of 20 J cm2. These findings suggest that right doses of PDT can maximize bacterial killing and neutrophil accumulation into the infected site. The doses lower than required dose result in insufficient killing of the bacteria and doses above the required dose damages host tissues [66].

There are different factors affecting the efficacy of MRSA infection *in vivo*. High dose light irradiation (210J/cm$^2$) causes complete resolution of MRSA in skin infection. Zolfaghari et al. even used visible light with MB as PS. Although the copious amount of light dose (360 J/cm$^2$ of laser light at 670 nm wavelength in the presence of 100 µg/ml methylene blue) cause 25-fold killing of MRSA which was not due to heat generated during treatments the temperature did not increase to more than 40$^o$C during 10 minutes exposure to the light. The histological examination showed that there is no cytotoxic effect *in vivo* by collateral tissue necrosis or architectural disturbance [67].

The effect of PDT by PS PPA904 [3, 7-bis(N, N-dibutylamino) phenothiazin-5-ium bromide] (Fig **4N**) in patients with bacterially colonized chronic leg ulcers and chronic diabetic foot ulcers was determined in a clinical trial by Morley et. al. The PPA904 or placebo was applied topically to wounds for 15 min, followed immediately by 50 J cm of red light. After three months the patients treated with PPA904 in chronic wounds demonstrated significant reduction in bacterial load compared to the placebo.

A large clinical trial (circa 6000 patients) was carried out in Vancouver, Canada to test whether nasal photodynamic disinfection mediated by MB plus red light decreased .S. aureus and MRSA in patients scheduled for elective cardiac, orthopedic, spine, vascular, thoracic and neurosurgical procedures [68, 69]. Patients received intranasal PDT in the preoperative area combined with chlorhexidine wipes on the skin. Nasal carriage was successfully reduced as measured by semi-quantitative colony counts in 83.9% patients colonized with MSSA, and in 87% of patients colonized with MRSA ($p=0.09$) immediately post-PDT. Importantly there was a 42% reduction in the post-surgical site infection rate compared to a four year historical average; this reduction was confirmed in an intent to treat analysis as well as with a propensity analysis of concurrent treated and untreated patients [70].

## MULTIDRUG EFFLUX SYSTEMS AND PDI

There are key bacterial phenotype elements that are under intensive interrogation as potential targets for photodynamic inactivation applications. Multidrug efflux has been identified as a major obstacle in efficient antimicrobial countermeasures. Microbial efflux pumps (MEP) are membrane transport systems that in addition to promoting resistance to antimicrobials, also perform essential roles in cellular metabolism. MEP exhibit activity in a wide range of organisms; they differ in membrane topology, energy coupling mechanisms, and most importantly in substrate specificities.[71-73]. MEP are classified into six super-families based on sequence and structural homology.[71] In Gram-positive bacteria and specifically *S. aureus* multidrug resistance is attributed to 3 chromosomally or plasmid encoded systems with broad and overlapping substrate specificity: Major Facilitator Superfamily (MFS), the multidrug and toxic compound extrusion (MATE) [74] and the Small Multidrug Resistance (SMR).[75]. The clinical role of efflux systems in resistance and emerging threats is the subject of inten-

sive research; they are implicated in MRSA [76], in Gram-negative pandrug resistant bacteria [77-79], and in *Mycobacterium tuberculosis* (MTB) [80]. The identification of substrates for MEP and the discovery of small molecule efflux pump inhibitors (EPIs) has been a rapidly expanding discipline. The NorA MFS system in *S. aureus* has historically been the most exploited target for discovery.

The role of efflux systems in PDT, the identification of PS-substrates and combinatorial treatment with EPIs has been studied indetail with the ABC mammalian transporters ABCG2 (Breast Cancer Resistance Protein BCRP), ABCC1 (multidrug resistance-associated protein 1, MRP1) and ABCB1 (P-glycoprotein, P-gp). [81-83], [84]. [85]. In brief an array of PS including porphyrins (phytoporphyrin, protoporphyrin IX) pyropheophorbides, purpurinimides and hypericin are substrates of ABCC1 and ABCG2 as their expression impaired PS efficacy for the majority of the chemotypes tested while the role of ABCB1 is not so clear. Efflux systems are characterized by substrate promiscuity. This promiscuity may explain the array of studies that have pinpointed porphyrins as efflux substrates. Porphyrins have been implicated in uptake and efflux by: 1) the Resistance Nodulation Cell Division (RND) AcrAB-TolC system in *E coli* [86]; 2) two co-regulated efflux transporters that modulate intracellular heme and protoporphyrin IX availability in *Streptococcus agalactiae*, [87]; and 3) a heme-regulated ABC transporter, HrtAB in *S. aureus* [88]. Although porphyrins have been used extensively in PDT applications for MRSA localized infections [89], the role of MEP in porphyrin mediated-PDT studies in *S. aureus* are contradictory. Grinholc et al [90] detected no efflux correlation in amphiphilic protoporphyrin diarginate PPArg mediated PDI when they employed a variety of efflux related *S. aureus* strains

The experimental evidence is more concrete for the phenothiazinium based PS MB and TBO. MB and TBO are amphipathic cations and share physicochemical similarity with the natural substrates of MFS efflux systems in Gram-positive bacteria like the antibacterial alkaloid berberine, [91, 92]. A model study employed a variety of efflux phenotypes in *S. aureus* and *P. aeruginosa* and compared phenothiazinium mediated PDI with the efficacy of other structurally unrelated PS. The results indicated regulation of the phenothiazinium PDI effect from the MFS NorA in *S. aureus* and a possible role for the RND MexAB in *P. aeruginosa* [93]. Further analysis with sixty clinical isolates of *P. aeruginosa* overexpressing efflux systems demonstrated that antibiotic-resistant *P. aeruginosa* was as susceptible to TBO-mediated PDI as susceptible strains [94]. Prates et al observed that ABC transporters and not MFS transporters affect MB-mediated PDT in the pathogenic yeast *Candida albicans* [93, 95]. *In silico* modeling studies, using MB and the crystal structure of the mammalian efflux system ABCB1 reveals a strong overlap for the PS binding site with the classic efflux substrates berberine and rhodamine 6G due to similarity in shape and electronic distribution [96]. The structurally related phenothiazines (thioridazine and chromazine) have been characterized as EPIs or functional transporter regulators as opposed to substrates of pathogenic efflux systems [97-99]. Taken together these observations provide evidence for the direct relation of MB and TBO with NorA. and the more perplexing interaction with the phenothiazinium chemical class.

## UVC IRRADIATION FOR MRSA INFECTIONS

Ultraviolet irradiation is a spectral-band of electromagnetic irradiation (wavelength: 100-400nm) divided into four discrete spectral areas comprising of UVA (315–400 nm), UVB (280– 315 nm), UVC (200–280 nm) and vacuum UV (100– 200 nm) [100]. UVC (termed germicidal UV) in the range of 262nm is the most lethal range of wavelength, as it gets absorbed by the DNA and RNA of the micro-organisms changing the structure of nucleic acids, resulting in dimerization of the pyrimidine molecules and formation of cyclobutane pyrimidine and pyrimidine pyrimidone dimers [101]. This dimerization distorts the DNA molecule, blocks the division and elongation of nucleic acids thus leading to cell death. Despite the well-known role of UVC irradiation as an antibacterial agent, its role to treat infections needs to be elucidated [102].

It is difficult to manage wound infections produced by multi drug resistant bacterial strains. A study by Conner-Kerr et al. observed the efficiency of UVC irradiation at a wavelength of 254nm, in inactivating antibiotic-resistant strains of *S. aureus* and *Enterococcus faecalis in vitro* [103]. Between 2 and 5 replications of bacterial suspensions at 10(8) CFU/ml were prepared and plated on sheep blood agar medium and exposed to UVC irradiation. MRSA killing rates were 99.9% at 5 s and 100% at 90 s and vancomycin-resistant *E. faecalis* (VRE), killing rates were 99.9% at 5 s and 100% at 45 s. Likewise another study by Rao *et al*. concluded that UVC irradiation had a good bactericidal effect for MRSA and *Streptococcus pyogenes* at a minimum time as 5 seconds and maximum t 15 seconds for MSSA and Enterococci species [104].

Buonanno et al. used an excimer lamp that produced monoenergetic UVC light irradiation at 207 nm with a filter to remove higher wavelength components. They described measurements of cell killing by 207-nm light, and compared the results with a conventional broad spectrum 254-nm germicidal UV lamp [105]. They measured the cell inactivation and DNA mutations in both for MRSA and also for normal fibroblasts cells and human skin tissue respectively. They observed that as compared to conventional UV lamp the 207nm UV light efficiently killed MRSA, and did not produce human cell killing. At doses of 207nm UVC that gave four-log level of MRSA killing, irradiation produced about 1, 000-fold less human cell killing i.e. it was not associated with mutations in the DNA as compared to conventional germicidal UV lamp. The study concluded that 207nm light irradiation efficiently kills the bacteria without causing any mutagenic effect in human cells, signifying that 207nm UV light irradiation may be a potential therapeutic application in infectious surgical site, including drug resistant bacteria [105].

Dai and coworkers have proposed that UVC (low pressure mercury lamp emitting 254-nm) may be a selective technique to kill bacterial and fungal pathogens including MRSA and MSSA in actual wound infections in mice without damaging the host tissue [106-108]. The potential of UVC light prophylaxis in highly contaminated superficial cutaneous wounds infected with bioluminescent P. aeruginosa and S. aureus was investigated on mouse model. The bacterial luminescence images of S. aureus-infected mouse skin abrasions with and without UVC light prophylaxis are shown in Figure A and B. The bacterial luminescence was completely eradicated on the wounds after exposure of UVC at 2.59 J/cm2. A lower reoccurrence of bacterial luminescence was observed after UVC light exposure (Fig. **6A**), whereas a strong bacterial luminescence remained until day 7 in the untreated group (Fig. **6B**). Biofilm formation in the wounds without UVC light prophylaxis was observed within 24 h after bacterial inoculation in Gram staining of the skin abrasions infected with S. aureus (Fig. **6C**). The mean bacterial luminescence of the mouse wounds treated by exposure to 2.59 J/cm2 UVC light at 30 min after bacterial inoculation from day 1 to day 10 (Fig. **6D**). The area under the curve of Fig D revealed the bacterial burden of the infected wounds was significantly reduced by UVC light (Fig. **6E**) [109].

There are a few clinical studies specifying that UVC can be detrimental to antibiotic resistant bacteria including MRSA in human patients. Thai et al. [110].published a case study involving three patients testing the potential role of UVC for the treatment of chronic wound ulcers infected with MRSA, in whom standard topical and oral antibiotics failed. UVC irradiation at 254 nm was applied to the wound for 180 seconds per wound site. This length of time was determined based on the MRSA killing rates reported in a previous *in vitro* study [103]. They reported that the UVC irradia-

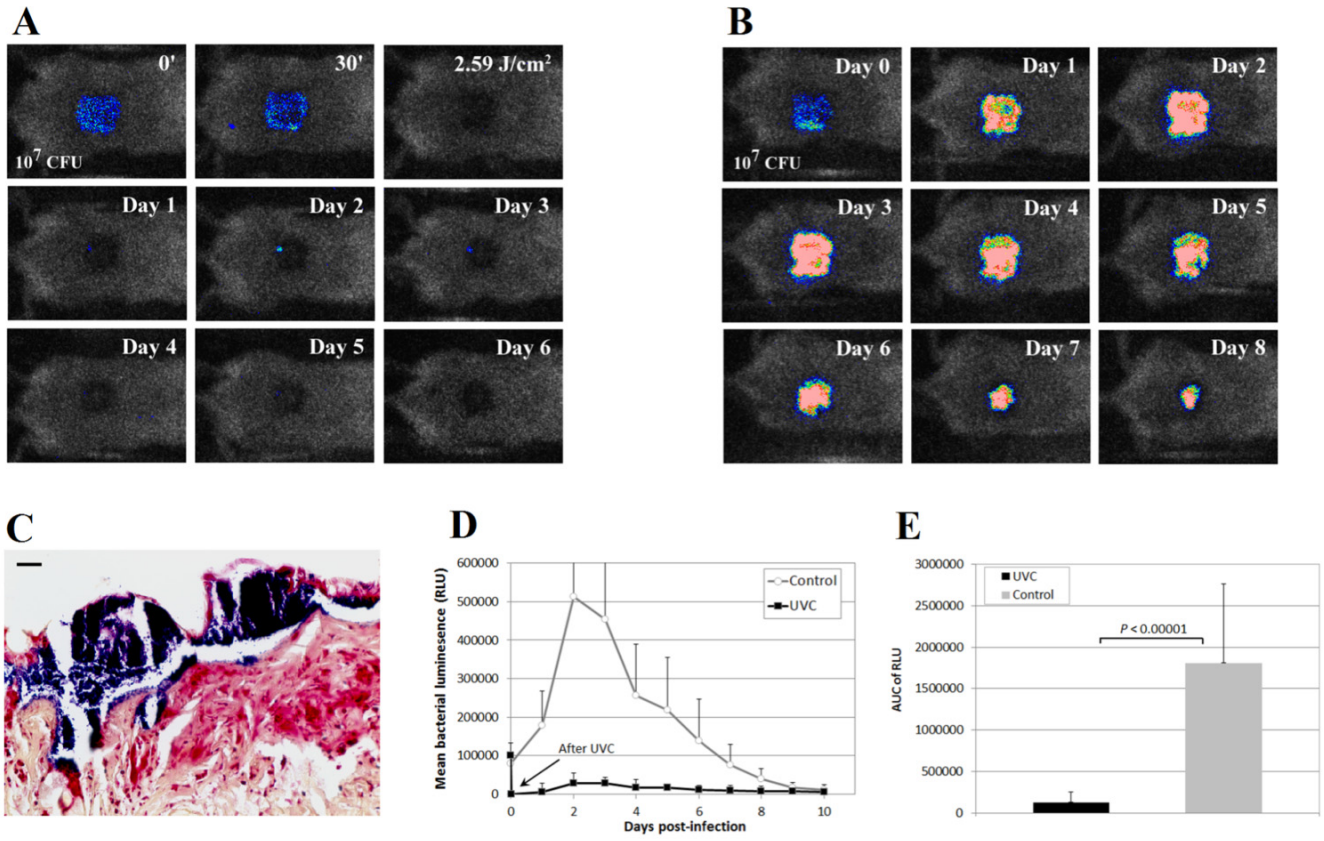


**Fig. (6).** (**A** and **B**) Successive bacterial luminescence images of representative mouse skin abrasions infected with 107 CFU of S. aureus with and without UVC light prophylaxis, respectively. **C**: S. aureus skin abrasion infection subjected to Gram staining. A biopsy sample was taken at 24 h after bacterial inoculation. Evidence of biofilm growth (blue) was observed in the wound. **D:** Time courses of mean bacterial luminescence of infected skin abrasions with (n=11) and without (n=12) UVC light prophylaxis. **E:** Mean areas under the bioluminescence-versus-time curves (in the two-dimensional coordinate system in panel D), representing the overall bacterial burdens of mouse wounds. Bars show standard deviations.

tion reduced bacterial load and facilitated the healing process, emphasizing the role of UVC irradiation as an adjuvant therapy for chronic wound infected with MRSA [110].

The same group later studied chronic ulcers exhibiting at least two signs of infections and critically colonized with bacteria in 22 patients [111]. The ulcers were irradiated once with UVC at a wavelength of 254nm for 180 seconds. Before and after the UVC irradiation semi-quantitative swabs were taken, to evaluate the changes in the bacterial bioburden within the wound bed. After UVC treatment, maximum decrease in the semi-quantitative swabs was observed in wounds colonized with the bacteria *Pseudomonas aeruginosa*. There was a significant reduction in the bacteria in the 12 ulcers infected with MRSA after UVC treatment [111].

## BLUE LIGHT FOR MRSA INFECTIONS

Blue light exhibits a broad-spectrum antimicrobial effect against both Gram-positive and Gram-negative bacteria without the addition of exogenous photosensitizers and it has been suggested as an alternative treatment modality for treating MRSA infections [112]. When compared to UV irradiation, blue light has been accepted to be much less detrimental to mammalian cells [113, 114].

Eradication of MRSA using blue light with 405-nm wavelength was investigated by Enwemeka et al. [115].and the results were promising. They found that consecutive delivery of two low doses of light was more effective than a single high dose. In another study, inactivation of Gram-positive bacteria like MRSA with blue light of 405 nm was found to be an oxygen-dependent process suggesting that the mechanism was photo-excitation of endogenous porphyrin molecules [116]. The type and concentration of porphyrins are different in various bacteria and accordingly different wavelengths may be required to be absorbed by various porphyrins. There is no exogenous PS involved in inactivation of bacteria using blue light which makes it easier to achieve. The wavelength of blue light used in the treatment of infection should be in the wavelength that is selectively absorbed by the chromophore located inside the pathogenic bacterial cells. For example blue light at a wavelength of 430nm failed to inactivate MRSA. So narrowband filters should be used for increasing the activity. Some Inactivation effects are observed at a wavelength of 420nm, however, the best activity was found at 405 nm. Blue light at this wavelength with an absolute dose of 23.5 J/cm$^2$ caused 2.4 log$_{10}$ reductions of MRSA [117].

Another study by Enwemeka's group tested two different strains of *S. aureus*; MRSA US-300 (strain of CA-MRSA) and the IS853 (strain of HA-MRSA) *in vitro* with different wavelengths of blue light. The results showed that various wavelength produced a statistically significant dose-dependent reduction in both strains. Maximum eradication of the CA-MRSA was achieved at 405 nm and HA-MRSA at 470 nm after 10 minutes irradiation. The eradication was also increased by increasing the light dose but this effect was not linear. The study concluded that photoinactivation with low dose blue light may be an effective clinical tool for MRSA infections [118].

Dai et al tested the efficacy of blue light (415 – 10 nm) therapy for eliminating community acquired MRSA infections in skin abrasions in a mouse model and infection was developed using bioluminescent USA300LAC: lux. Figure **7A** shows the successive bio-

luminescence images of a representative mouse skin abrasion infected with USA300 LAC: : lux and treated with(top row) or without (bottom row) blue light at 30 min after bacterial inoculation. The bacterial luminescence was eliminated after 41.4 J/cm2 blue light had been delivered (46 min illumination at the irradiance of 15.0 mW/cm2) compared to untreated control wound, in which the bacterial luminescence almost remained unchanged during the same period of time. Figure **7B** shows quantification of the relative luminescence units for mice treated with blue light and mice kept in the dark for the same time period. The blue light values were significantly less than dark values at all fluences tested. They concluded that there existence of a therapeutic window of blue light for bacterial infections where bacteria are selectively inactivated while host tissue cells are preserved [119]. The underlying mechanism of action is proposed to be the photoexcitation of endogenous intracellular porphyrins leading to ROS generation, that is toxic to bacterial cells [120-122]. Blue light is already being used clinically for treatment of inflammatory acne caused by *Propionibacterium acnes* [123-126]. Clinical trials have been conducted to investigate the use of blue light for *Helicobacter pylori* stomach infections and promising results have been achieved [127, 128]. It was also shown to be effective in acute, potentially lethal *Pseudomonas aeruginosa* burn infections in mice [129]. However the majority of the studies on the antimicrobial effect of blue light, especially for MRSA infections have been confined to *in vitro* studies [130-132].

As compared to UV, there is less concern about the mutagenic effects of blue light in mammalian cells as blue light is not absorbed by DNA. Although penetration of blue light is deeper into tissue than UV light, there may be need for approaches trying to increase its penetration. Since the microbial cells can develop some resistance to UV irradiation, it would be worthy to consider whether resistance can be induced to blue light in microbial cells [133]. In studies with aPDT, tens or hundreds fold higher fluences of light exposures were required for the equivalent bacterial inactivation *in vivo* than *in vitro* [63, 134, 135] . One possible explanation may be that *in vivo* environment possibly favored the metabolism of bacterial cells compared with broth cultured cells, and promoted the biosynthesis of intracellular porphyrins, making the bacteria more photosensitive.

## NEAR INFRARED IRRADIATION FOR MRSA INFECTIONS

In order to avoid the potential DNA-damaging effects of UVC to mammalian tissues, the use of near-infrared (NIR) as an antimicrobial therapy has been suggested as an alternative treatment for MRSA infections [136]. Studies have demonstrated that even though photodamage to several pathogens were achieved at doses that did not give increases above physiologic temperatures, no observable damage was observed to mammalian cells or tissues [137, 138]. Chromophores absorbing the NIR range energy that are present in all cells are proposed to be the C-H covalent bonds of phospholipids in the plasma and mitochondrial membranes and cytochrome c in the electron transport chain [137]. Bornstein et al. investigated the effects of a multiplex dual wavelength NIR (870 and 930 nm) *in vitro* and *in vivo* on several bacterial and fungal pathogens including erythromycin resistant MRSA with a calculated nonlethal dosimetry and reported a decrease in transmembrane potentials across the plasma membrane and across the mitochondria as well as an increase in ROS generation [137]. While a decrease in

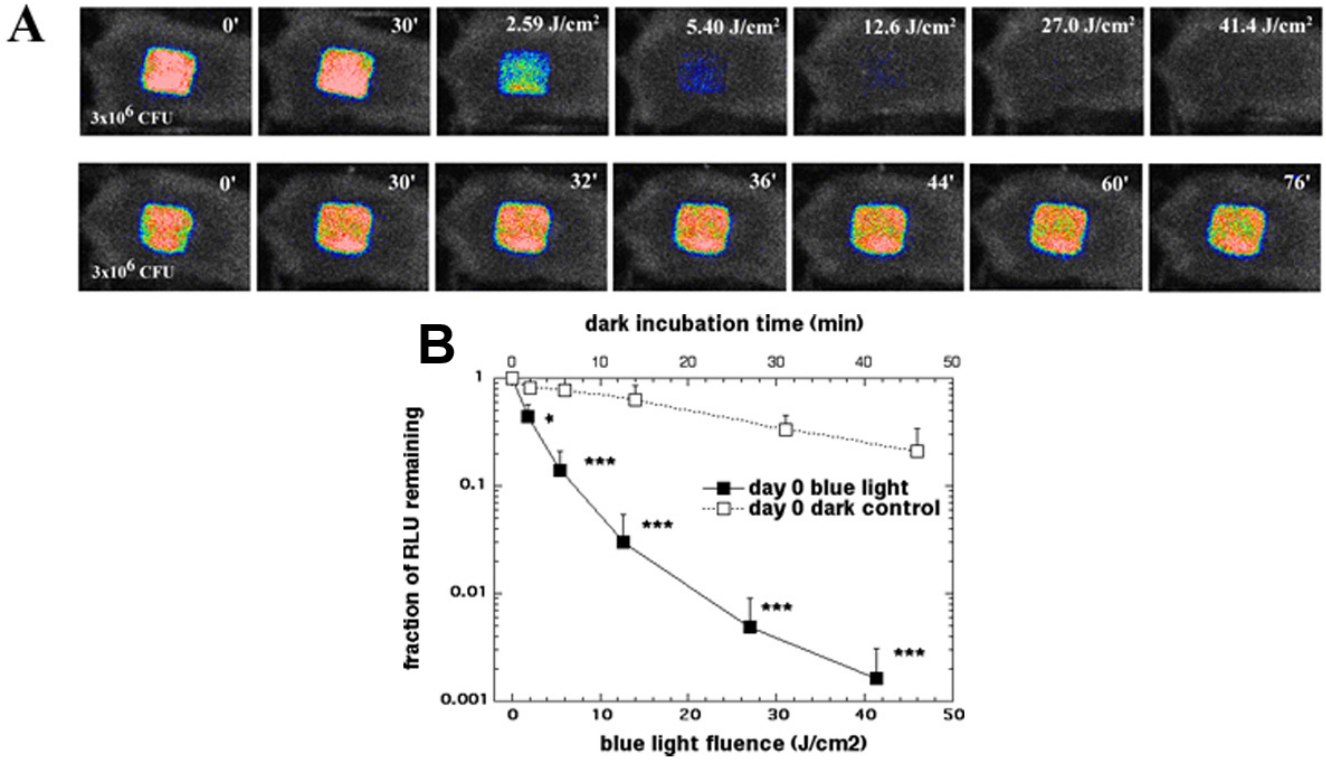


**Fig. (7).** Blue light prophylaxis and treatment of community-acquired methicillin-resistant Staphylococcus aureus (CA-MRSA) infections in mouse skin abrasions. **A:** Successive bacterial luminescence images of a representative mouse skin abrasion infected with 3 ·X $10^6$ colony-forming units (CFU) of USA300 LAC::lux and exposed to 41.4 J/cm2 blue light at 30 min (day 0) after bacterial inoculation (top row). Corresponding images of a MRSA infected representative mouse skin abrasion kept in the dark for the amount of time necessary to deliver blue light to the treated mice. **B:** Dose response curves showing mean fraction of luminescence remaining from both blue light treated mice (n = 4) and dark control mice (n = 4). Bars are SD. *p < 0.5; ***p < 0.001 versus dark controls.

plasma membrane potential (Psip) was observed in Gram-positive and Gram-negative bacteria, a decrease in mitochondrial membrane potential (Psimt) was observed in pathogenic fungi. In one clinical part of this study, NIR was applied on human carriers intranasally, around the anterior nares and the results demonstrated complete clearance of erythromycin resistant MRSA by triplicate culture in all three carriers following the second laser treatment on day 3. Moreover it is worthwhile to note that all three cultures remained clear through day 5. In a subsequent study, the same group investigated whether photodamage could potentiate the antibacterial effects of certain conventional antimicrobials in MRSA infections [139]. MRSA (Strain HSJ216) and fluoroquinolone-resistant MRSA were tested *in vitro* with same wavelengths but this time with sub-inhibitory concentrations of erythromycin, tetracycline, penicillin, rifampin and trimethroprim and with erythromycin and tetracycline respectively. 97% potentiation was achieved with erythromycin and tetracyclin and it was suggested that this antibiotic potentiation probably resulted from a photobiological attenuation of ATP-dependent macromolecular synthetic pathways, similar to that observed with daptomycin, via disruption of (DeltaPsip) and endogenous generation of ROS. Another interesting observation was that the staphyloxanthin carotenoid pigment located in the cell membrane of *S. aureus* that functions as an antioxidant, was providing protection against the photo-generated ROS. On the other hand, with erythromycin, tetracycline and ciprofloxacin, attenuation of energy-dependent efflux systems also seems to be another possibility. Based on these studies, NIR treatment of MRSA infections may be suggested as a complementary treatment in order to enhance the therapeutic potential of antibiotics.

## CONCLUSION

Considering the apparently unstoppable progress of antibiotic resistance, it is likely that non-antibiotic approaches will assume an increasingly important role in therapy. Among these non-antibiotic approaches we believe that light-based antimicrobial technologies will play a major part. The lack of demonstrated induction of resistance to light based approaches (with the possible exception of UVC) is encouraging. Gram-positive bacterial species (especially Staphylococci) appear to be among the easiest species to kill by light. Most of the studies that have compared MRSA to MSSA have found that there is little difference in susceptibility to photoinactivation between the strains. As mentioned previously, the chief obstacle to be overcome is the limited penetration of light into tissue. However several different approaches have been developed to overcome this limitation. Optical clearing employs harmless substances such as glycerol that match the refractive index of tissue (1.5-1.6) to dramatically reduce the effects of tissue scattering of light [140]. Optical fibers with a very narrow diameter (less than 100 micron) have been developed that can be inserted into the tissue in a minimally invasive manner to deliver light to almost any depth [141] and the light distribution within the tissue is being studied using the techniques of tissue optics particularly Monte Carlo modeling [142]. Finally the technique of two-photon excitation employs a short pulsed laser (femtoseconds) to deliver photons at twice the wavelength that is desired to be absorbed [143]; for instance two 800 nm photons arriving at the chromophore at the same time can behave as if they were a single 400 nm photon. Although at present this technique requires a relatively expensive titanium sapphire laser, newer light sources are expected to be developed in the coming years.

## CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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