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Light-excited antibiotics for potentiating bacterial killing via reactive oxygen species generation

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KEYWORDS: light irradiation, antibiotics, photodynamic antibacterium, reactive oxygen species, photosensitizer

ABSTRACT: The irrational or excessive use of antibiotics causes the emergence of bacterial resistance, making antibiotics less effective or ineffective. As resistant antibiotics increase, it is crucial to develop new strategy and innovative approaches to

potentiate the efficacy of existing antibiotics. In this paper, we report that some existing antibiotics can produce reactive oxygen species (ROS) directly under light irradiation. Thus, a novel antibacterial photodynamic therapy (PDT) strategy is proposed by using the existing antibiotics whose activities are potentiated via light-activation. This antibiotic-based PDT strategy can achieve the efficient bacteria killing with low dosage of antibiotics, indicating that bacterial killing can be enhanced by the light irradiated antibiotics. Moreover, the specific types of ROS produced by different antibiotics under light irradiation were studied for better elucidation of the antibacterial mechanism. The findings can extend the application of the existing antibiotics and provides a promising strategy for treatment of bacterial infections, and even cancers.

Introduction

Since the discovery of penicillin in 1929, antibiotics have been widely used in the treatment of infectious diseases, helping humans to reduce the threat of bacterial infections.[1] However, the irrational or excessive use of antibiotics has given rise to a large number of drug-resistant bacteria such as methicillin-resistant *staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), and multidrug-resistant *A. baumannii* (MRAB).[2-5] The emergence of bacterial resistance causes antibiotics to become less effective or ineffective against drug resistant pathogens. Antibiotic resistance has become a grave and growing public health problem, and poses a serious threat to human health. Especially, the emergence and the rapid spread of drug-resistant bacteria make common infections difficult to be cured with the existing antimicrobial drugs. Eventually, there will be no appropriate drugs to treat an even common disease and minor injuries.[6-8]

To date, although antimicrobial materials and new antimicrobial strategies have been studied to kill bacteria,[9-13] antibiotics remain the most commonly used antimicrobial drugs in clinical practice. Scientists around the world are trying to develop the new

antibiotics, which is the most fundamental way to overcome current antibiotic resistance.[14] However, because the synthesis and clinical trials of new antibiotics take a long time, the development of new antibiotics lags far behind the production rate of drug-resistant bacteria.[8,14] In contrast, the existing antibiotics have the advantages of large quantity, low cost, and clear antimicrobial mechanism, although the efficacy of some existing antibiotics has gradually declined with the development of drug-resistant bacteria.[15,16] Therefore, the development of new methods and strategies to potentiate the efficacy of existing antibiotics have attracted much attention.[17-19]

Reactive oxygen species (ROS) are a group of active molecules containing oxygen, including hydroxyl radicals (•OH), superoxide radical ($\bullet O_2^-$), and singlet oxygen ($^1O_2$), etc. Depending on photosensitizers or materials to produce ROS under appropriate light irradiation, photodynamic therapy (PDT) strategy was demonstrated to carry out bacteria killing.[20-24] The ROS produced by light irradiation can rapidly damage nearly all types of biomolecules such as proteins, lipids, and nucleic acids, resulting in bacterial death. Therefore, PDT has the rapid and broad-spectrum antimicrobial properties and is

usually not easy to produce resistance in bacteria.[25,26] Furthermore, studies have shown that different kinds of antibiotics can kill bacteria not only through their specific mechanisms of action, but also through the activation of cell metabolisms or the synergistic interaction between antibiotic and materials to produce ROS.[27-30] Courtney et al reported that a light-activated quantum dot (QD) nanoparticle could produce specific ROS, superoxide, and potentiated the activity of antibiotics in drug-resistant clinical isolates.[30] However, the QD nanoparticles are usually limited in wide application due to their inherent toxicity and poor biocompatibility. It was reported that ROS could be produced directly by tetracycline antibiotics under light irradiation with ultraviolet light (360 nm) or blue light (415 nm).[31,32] The tetracycline antibiotics acted as photosensitizers to perform antimicrobial PDT and potentiate the bacterial killing. Thus, the light irradiation provides an important way to improve the antibacterial ability of antibiotics. However, other antibiotics with photodynamic activity are rarely reported among the existing antibiotics.

Herein, we demonstrate that some existing antibiotics can directly produce ROS under light irradiation to achieve the antibacterial PDT. Although the bacteria can resist the common antibiotics with non-lethal dose, the antibacterial activity of these antibiotics can be enhanced under light irradiation through ROS generation, leading to the enhancement of the bacteria killing. This PDT approach reduces the therapeutic dose of these antibiotics, thereby reducing the emergence of resistance. This antibiotic-based antibacterial PDT strategy differs fundamentally from previously described antibacterial technology in several respects. First, the antibiotic used as photosensitizer for antibacterial PDT is simple, cost-effective, and easy to be obtained from commercial channels. Combination of their inherent antimicrobial mechanism and photodynamic activity, the resistant antibiotics can be revived with their enhanced antibacterial activity. Second, the visible light source used in the light-excited antibiotics further reduces the risk of bacterial damage caused by the light itself as compared with ultraviolet and blue light sources. Third, since the toxicological and pharmacological properties of existing antibiotics have been clearly studied, the development of antibiotic-based antibacterial

PDT can provide a rapid, efficient, and biocompatible way for potentiating bacterial killing.

Results and discussion

The ability of different antibiotics to produce ROS

We investigated the ROS-generating ability of six types of antibiotics, including polypeptide (polymyxin B, PMB), sulfonamide (sulfamethizole, SMT), fluoroquinolone (norfloxacin, Norf), glycopeptide teicoplanin (Teic), beta-lactam (ampicillin, AMP), and aminoglycoside (kanamycin, Kana). The structures of these antibiotics were shown in Figure 1a. A fluorescence dye 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA), which is non-fluorescent and become fluorescent 2′,7′-dichlorodihydrofluorescein (DCF) nonspecifically in the presence of a variety of ROS,[33] was used to measure the total ROS produced by antibiotics. As shown in Figure 1b, there are weak fluorescence responses of DCF with the antibiotics without light irradiation. However, the strong fluorescence peaks of DCF at 525 nm are obviously observed from the four antibiotics PMB, SMT, Norf, and Teic solution under light irradiation. These results indicate that the

four antibiotics can produce ROS under light irradiation, which provides the potential for enhancing the antibacterial activity. For Kana and AMP with DCFDA, there are no apparent fluorescence enhancements under light irradiation (Figure S1a, S1b). These results indicate that Kana and AMP cannot produce ROS and their antibacterial activities cannot be enhanced under light irradiation so they are not suitable for antibacterial PDT.

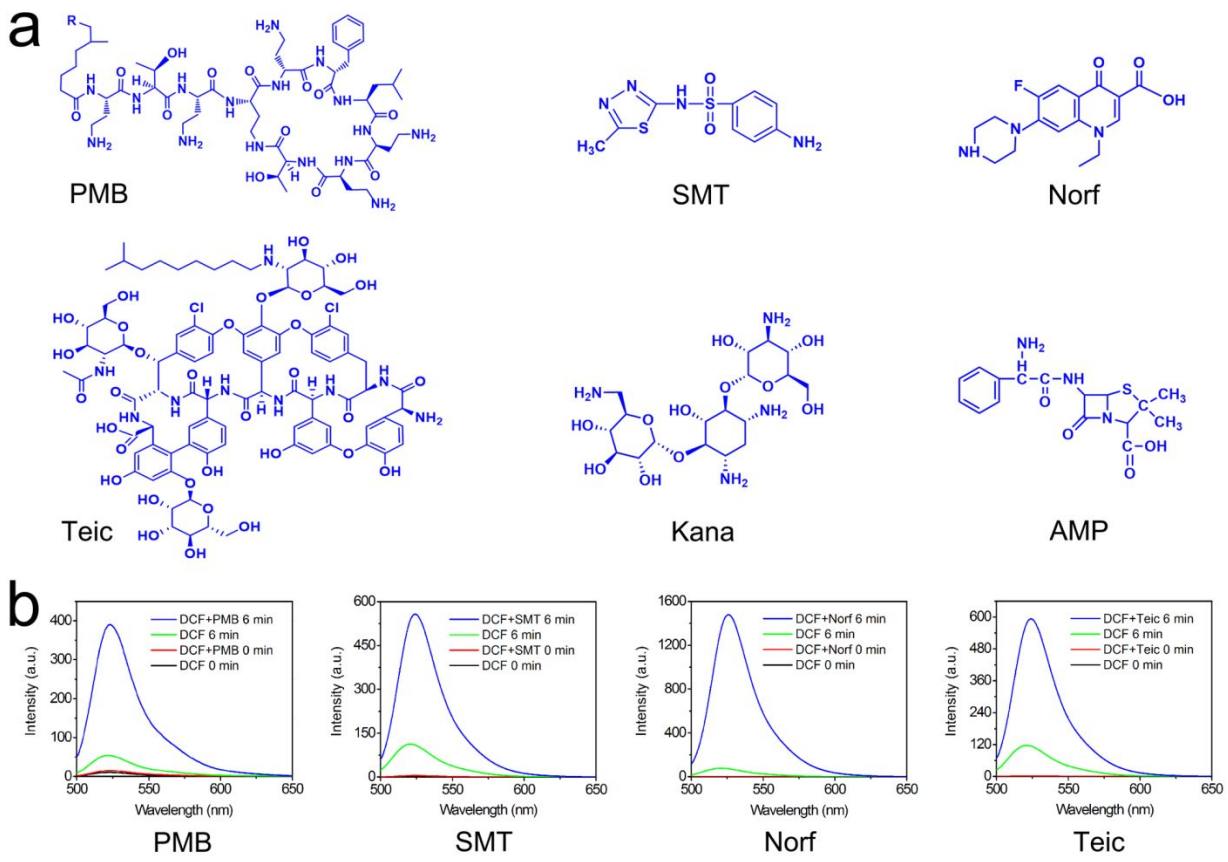
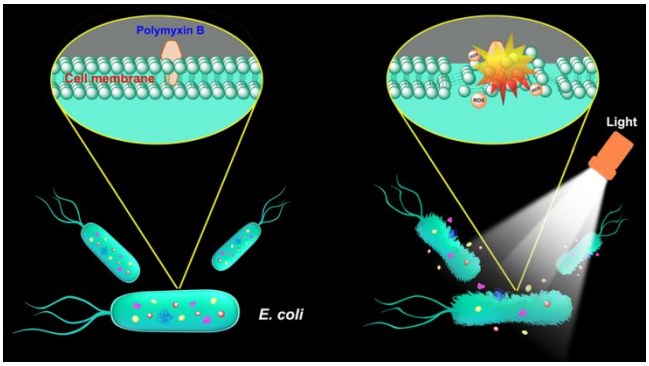


Figure 1. The structures of the antibiotics and total ROS detection. (a) Summary of the structures of the used antibiotics. (b) Detection of ROS from different antibiotics by fluorescence dye DCFDA.

Light irradiated antibiotics for enhancing the killing of gram-negative bacterium

To test the antibacterial effect of the light-excited antibiotics, we selected antibiotic PMB and a gram-negative bacterium, *Escherichia coli* (*E. coli*, BL21) with kanamycin resistance (kana^r *E. coli*) as research model. Before studying the killing effect of PMB,

we demonstrated that Kana has no effect on the viability of kana^r *E. coli* either in the dark or in the light because the *E. coli* used is resistant to Kana and it can not to produce ROS (Figure S2a). PMB as a polypeptide antibiotic primarily binds to the gram-negative bacteria through electrostatic interactions and usually achieves the bacterial killing by altering the permeability of bacterial outer membrane.[34] In this work, light irradiated PMB is tested to potentiate the bacterial killing. We first used DCFDA staining to study intracellular ROS production in PMB-treated kana^r *E. coli* by confocal laser scanning microscope (CLSM) imaging. As shown in Figure 2a, there is almost no green fluorescence from DCF in kana^r *E. coli* under light irradiation in the absence of PMB. In contrast, the distinct green fluorescence from DCF is observed in kana^r *E. coli* treated with PMB under light irradiation, suggesting that ROS can be produced from PMB bound to kana^r *E. coli*.



Scheme 1. Schematic illustration of light excited antibiotic PMB producing ROS to potentiate the kana^r *E. coli* killing.

A schematic illustration of light excited PMB for enhancing the killing of kana^r *E. coli* is shown in Scheme 1. PMB with non-lethal dose has little damage on the kana^r *E. coli* without light irradiation. The PMB, however, produces ROS under light irradiation, which can kill the kana^r *E. coli*. Figure 2b shows the bacterial viability of kana^r *E. coli* treated by PMB without and with light irradiation, respectively. The viability of kana^r *E. coli* declined slightly with the concentration of PMB from 1 to 4 μg/mL and decreased observably from 4 to 8 μg/mL in the dark. The 90% inhibitory concentration (IC90) of PMB against kana^r *E. coli* was acquired as 7.8

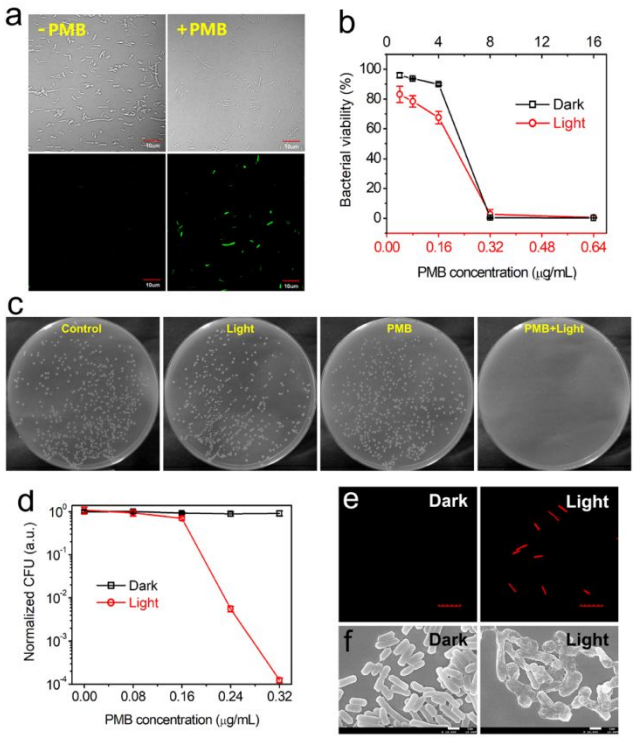


Figure 2. Potentiating antibiotic in killing of gram-negative bacterium kana^r *E. coli* via light irradiation. (a) Intracellular detection of ROS production using DCFDA staining. (b) The viability of kana^r *E. coli* treated with PMB in the dark (top axis) and in the light (bottom axis). Error bars represent standard deviation from three repeated measurements. (c) Plate photographs for kana^r *E. coli* diluted by the same times after treatment without light (Control), with light, PMB alone, and PMB+light, and respectively. PMB: 0.32 μg/mL. (d) The CFU values of kana^r *E. coli* treated by PMB in the dark and in the light. Light irradiation with 50 mW/cm^2 for 40 min. (e) CLSM and (f) SEM images of

bactericidal effect of PMB against kana^r *E. coli* treated with 0.64 μg/mL PMB in the dark and in the light. Light irradiation with 40 mW/cm^2 for 40 min. Scale bar: 10 μm in CLSM images and 1 μm in SEM images.

μg/mL in the dark (Figure 2b, Table S1). By comparison, the viability of kana^r *E. coli* reduced significantly with the concentration of PMB from 0.04 to 0.32 μg/mL under light irradiation (Figure 2b, Table S1). The optimal light irradiation time and light intensity for the light-activated PMB to kill kana^r *E. coli* were shown in Figure S3. The IC90 value of PMB against kana^r *E. coli* was reduced to 0.3 μg/mL in the light, which was 26 times lower than that of 7.8 μg/mL in the dark. These results indicate that the antibacterial activity of PMB can be obviously potentiated through light-activation.

To verify the enhanced antibacterial activity of light-excited PMB, we utilized the colony-forming units (CFU) counting method to investigate the antibacterial efficacy of PMB. As can be seen from Figure 2c, the survival rates of kana^r *E. coli* treated with light irradiation only (Figure 2c, Light) or low-dose PMB alone (Figure 2c, PMB) are not significantly different from those treated without any treatment (Figure 2c, Control).

However, when the same low dose of PMB is used to treat the kana^r *E. coli* with light irradiation (Figure 2c, PMB+Light), the survival rate of *E. coli* reduces to nearly 1%. Figure 2d shows that the CFU value of kana^r *E. coli* treated by PMB with 0.32 μg/mL decreases 3.7 orders of magnitude in the light compared to in the dark, indicating that the light-excited PMB can further damage the bacterial cells and enhance the bacteria killing.

To evaluate the antibacterial effect of light-activated PMB, CLSM imaging was used to directly observe the antibacterial effect of PMB against the kana^r *E. coli* treated with or without light irradiation. As shown in Figure 2e, when *E. coli* is treated with PMB in dark, there is no red fluorescence emission of propidium iodide (PI), which is a DNA stain to evaluate cell viability. The result can be attributed to the fact that the kana^r *E. coli* resists the low dosage of PMB (0.64 μg/mL), which is not sufficient to kill the kana^r *E. coli*. However, for the kana^r *E. coli* treated with PMB and light irradiation together (Figure 2e), the red fluorescence of PI is observed in fluorescence field. This is because that PMB binds to the cell membrane of *E. coli* through the electrostatic interaction and produces ROS under light irradiation, leading to the bacteria death. Moreover, scanning electron

microscope (SEM) was used to study the microscopic structure of *E. coli* for investigating the bactericidal effect. As shown in Figure 2f, SEM imaging shows that the morphology and membrane structure of *E. coli* are unbroken for the *E. coli* treated with PMB in dark, because the dosage of PMB with 0.64 μg/mL is extremely low and a non-lethal dose. By contrast, the *E. coli* treated with both PMB and light irradiation is ruptured and collapsed, indicating that the *E. coli* has been killed owing to the leakage of intracellular contents. The microscopic characteristics of *E. coli* observed by SEM further confirm that the light-excited PMB can greatly damage the cell membrane and improve its antibacterial activity against kana^r *E. coli*, which is consistent with the result of CLSM.

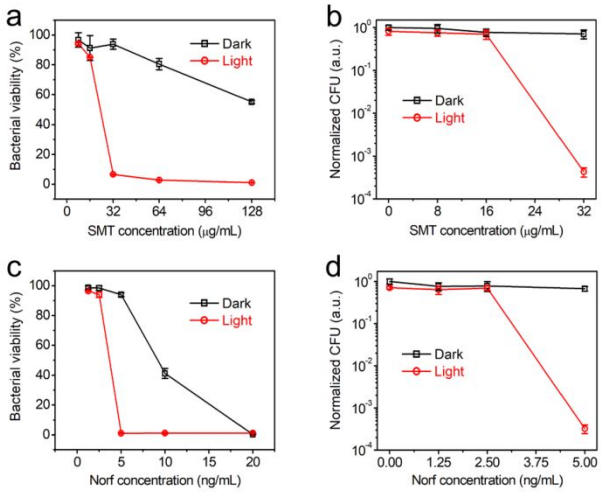


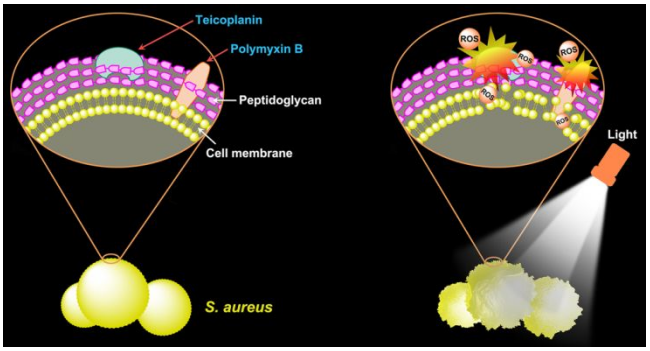
Figure 3. Potentiating SMT and Norf in killing of gram-negative bacterium kana^r *E. coli* via light irradiation. The viability of kana^r *E. coli* treated with SMT (a) and Norf (c) in the dark and in the light, respectively. (b) The CFU values of kana^r *E. coli* treated with SMT (b) and Norf (d) in the dark and in the light, respectively. Light irradiation with 50 mW/cm^2 for 40 min. Error bars represent standard deviation from three repeated measurements.

To further verify the potentiation of other light-activated antibiotics, we investigated the other three types of antibiotics, including SMT, Norf, and AMP that were usually used against gram-negative bacteria. SMT usually interferes with folate metabolism, resulting in bacteria death.[35] However, as shown in Figure 3a, the low concentration of SMT with

non-lethal dose cannot kill the kana^r *E. coli* in the dark (black curve). SMT can induce the killing of the kana^r *E. coli* by producing ROS under light irradiation (red curve). When the concentration of SMT increases from 8 to 128 μg/mL without light irradiation, the corresponding viability of kana^r *E. coli* slightly decreases from 96.7% to 55.1%. In contrast, the viability of kana^r *E. coli* under light irradiation is gradually reduced from 94.1% to 6.6% with the increasing of the concentration of the SMT from 8 to 32 μg/mL. The IC90 value of SMT against kana^r *E. coli* is reduced from >128 to 31.5 μg/mL (Figure 3a, Table S1). The CFU value of kana^r *E. coli* treated by SMT with 32 μg/mL decreases by 3.2 orders of magnitude in the light compared to in the dark (Figure 3b), indicating that the killing efficiency of SMT in the light enhances remarkably as compared with in the dark. Similarly, although quinolone antibiotic Norf can bind to topoisomerase and interfere with the DNA synthesis,[35,36] it is not enough to kill bacteria with low dosage (Figure 3c). The enhanced antibacterial activity of Norf against kana^r *E. coli* is acquired under light irradiation as compared with those of Norf without light irradiation (Figure 3c, 3d). Accordingly, the IC90 value of Norf against kana^r *E. coli* is reduced from 17.6 to 4.8 ng/mL (Figure 3c, Table S1). The CFU value of kana^r *E. coli*

treated by Norf with 5 ng/mL decreases by 3.3 orders of magnitude in the light compared to in the dark (Figure 3d). These results show that the light-activation can potentiate the antibacterial activity of SMT and Norf for killing *E. coli*. However, the beta-lactam antibiotic AMP with different concentrations has almost the same effect on the viability of kana^r *E. coli* with and without light irradiation (Figure S2d). The result indicates that light-activation has no effect on the antibacterial activity of AMP against *E. coli*, which is attributed to the fact that AMP is unable to produce ROS under light irradiation.

Light irradiated antibiotics for enhancing the killing of gram-positive bacterium



Scheme 2. Schematic illustration of the photoexcited Teic and PMB producing ROS to potentiate the *S. aureus* killing.

In order to further test the potentiation of light-activated antibiotics, we selected a gram-positive bacterium *Staphylococcus aureus* (*S. aureus*) and glycopeptide antibiotic Teic to investigate the photodynamic killing of *S. aureus*. Generally, Teic inhibits peptidoglycan synthesis by binding the D-alanyl-D-alanine dipeptide in bacterial membrane and blocking transglycosylase activity for killing of bacteria.[37] The schematic diagram of antibacterial PDT mechanism of Teic against *S. aureus* is shown in Scheme 2. Teic bound to *S. aureus* with low concentration is not enough to kill *S. aureus*. The *S. aureus*, however, may be killed by ROS produced by Teic under light irradiation. As can be seen from Figure 4a, the IC90 value of Teic against *S. aureus* treated with light irradiation is 0.31 μg/mL, which is 3.9 times lower than that of 1.22 μg/mL treated without light irradiation. Figure 4b is the CFU counting method to evaluate the photodynamic bactericidal efficacy of Teic. The CFU counting result of *S. aureus* treated with light irradiation only (Figure 4b, Light) or Teic alone (Figure 4b, Teic) is almost the same as that of *S. aureus* without any treatment (Figure 4b, Control), which indicate that the light irradiation only or low dose of Teic alone can hardly kill the *S. aureus*. By contrast, the low doses of Teic are nearly 98% effective in killing *S. aureus* under light

irradiation (Figure 4b, Teic + Light). Figure 4c shows that the killing efficiency of Teic with 0.32 μg/mL against *S. aureus* was 4.9 orders of magnitude higher in the light than in the dark, demonstrating that the bactericidal activity of Teic against gram-positive bacterium can be potentiated via light-activation.

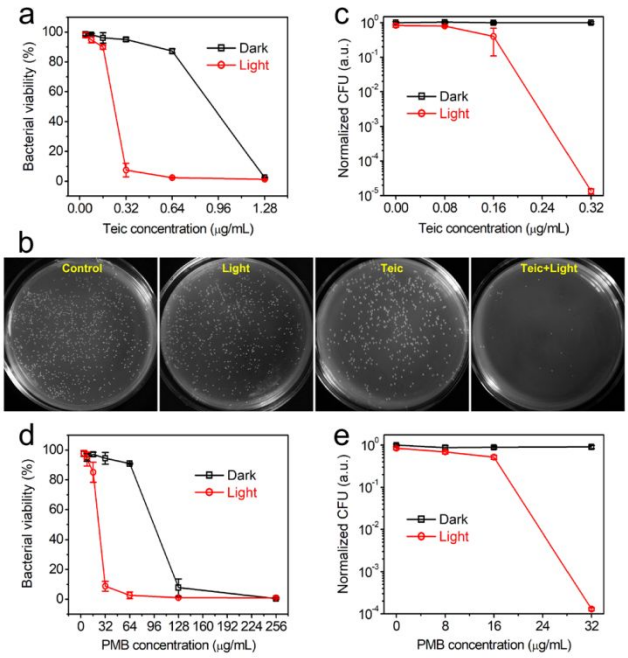


Figure 4. Potentiating antibiotics in killing of gram-positive bacterium *S. aureus* via light irradiation. The viability of *S. aureus* treated with Teic (a) and PMB (d) in the dark and in the light, respectively. Error bars represent standard deviation from three repeated measurements. (b) Plate photographs for *S. aureus* diluted by the same times after treatment without light (Control), with light, Teic alone, and Teic+light, and respectively.

Teic concentration: 0.32 μg/mL. The CFU values of *S. aureus* treated by Teic (c) and PMB (e) in the dark and in the light, respectively. Light irradiation with 40 mW/cm^2 for 40 min.

Commonly, narrow-spectrum antibiotics are effective only against specific classes of target pathogens.[38] Among them, PMB is mainly suitable for gram-negative bacteria owing to its ability for specifically binding to the cell membrane. PMB is not normally used to treat gram-positive bacteria because the cell walls of gram-positive bacteria with peptidoglycan are too thick to bind with PMB easily. However, the photodynamic antibacterial activity of PMB inspired us to study the light-activated PMB against gram-positive bacterium. The mechanism of the light-activated PMB for the killing of *S. aureus* is shown in Scheme 2. Despite a small amount of PMB that binds to *S. aureus* cannot damage the *S. aureus* in the dark, it can achieve the killing of *S. aureus* by producing ROS with light irradiation. As shown in Figure 4d and 4e, the killing effect of PMB on *S. aureus* is significantly higher under light irradiation than without light irradiation. The IC90 value of PMB against *S. aureus* is reduced from 125.5 μg/mL without light

irradiation to 31.0 μg/mL with light irradiation (Figure 4d, Table S1). The killing efficiency of PMB against *S. aureus* improves by 3.9 orders of magnitude in the light as compared with in the dark (Figure 4e). These results indicate that the antibacterial activity of PMB against *S. aureus* can be enhanced by ROS produced by PMB under light irradiation, which provides a possibility for the colistin antibiotics against gram-positive bacteria. At the same time, it also shows that the antibiotic-based PDT can expand the application of antibiotics.

Specific ROS characterization by fluorescent indicators and EPR spectrum

In antimicrobial PDT, the ROS that play a bactericidal role mainly involve three types, including •OH, $•O_2^-$, and $^1O_2$.[21] To better understand the PDT mechanism of the light-excited antibiotics, we systematically investigated the ability of PMB, SMT, Norf, and Teic to produce the specific type of ROS, including •OH, $•O_2^-$, or $^1O_2$. These specific ROS were measured by using specific ROS fluorescence dyes and electron paramagnetic resonance (EPR) spectrum, respectively.

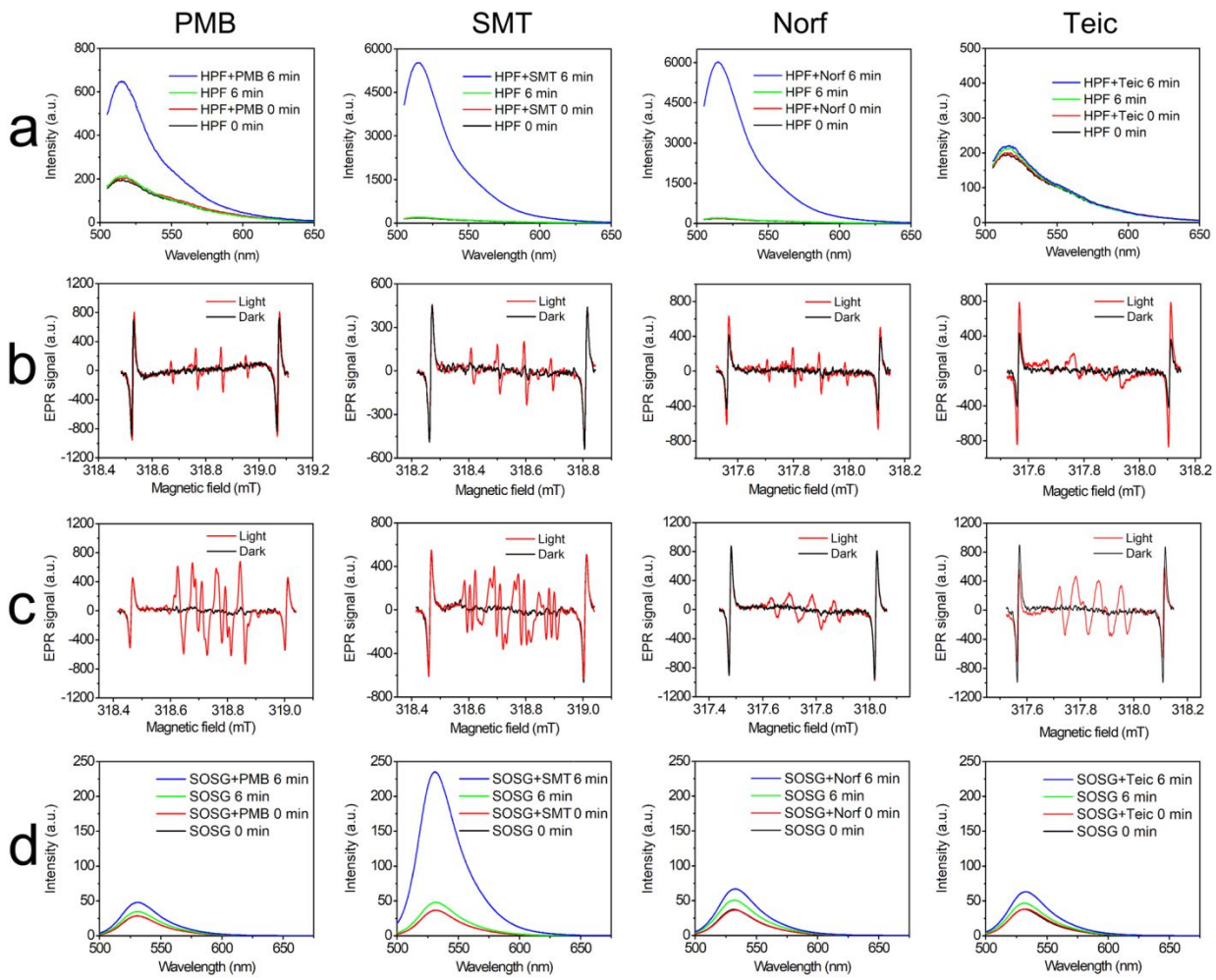


Figure 5. Detection of the specific type of ROS. (a) Detection of •OH from different antibiotics by fluorescence dye HPF. EPR spectra of (b) DMPO-•OH adducts and (c) DMPO-•O_2^- adducts. (d) Detection of $^1O_2$ from different antibiotics by fluorescence dye SOSG.

A specific fluorescence dye 3′-(p-hydroxyphenyl) fluorescein (HPF) was used to detect •OH.[39] As shown in Figure 5a, the fluorescent signals are weak in the solution of

the four antibiotics with HPF in the dark. In contrast, the strong fluorescent signals are observed in the solution of three antibiotics PMB, SMT, and Norf with HPF under light irradiation. Because HPF itself is non-fluorescent and emits fluorescence through interaction with •OH specifically, we infer that these three antibiotics produce the •OH under light irradiation. However, the fluorescence signal of HPF with Teic is weak with irradiation, suggesting that the ability of Teic to produce •OH under light irradiation is poor. Moreover, the •OH was further characterized by using EPR spectra with 5,5-Dimethyl-1-pyrroline-n-oxide (DMPO) as the trapping reagent.[40] As shown in Figure 5b, four-line EPR characteristic signal of DMPO-•OH spin adduct of the antibiotics PMB, SMT, and Norf are clearly observed in the EPR spectra. The intensity ratios of the four-line signal are 1: 2: 2: 1, indicating that •OH is generated in the antibiotics solutions under light irradiation. For Teic, there is no evident EPR characteristic signals from •OH in the EPR spectra, suggesting that Teic has the poor ability to produce •OH under light irradiation. These detection results of •OH by EPR are consistent with those of fluorescence detection by HPF.

The $\bullet O_2^-$ production of the four antibiotics under light irradiation was detected by using the EPR spectrum.[40] As shown in Figure 5c, Teic with light irradiation has the EPR characteristic signal of DMPO-$\bullet O_2^-$ spin adduct with intensity ratios of 1:1:1:1, indicating that Teic can produce $\bullet O_2^-$ under light irradiation. For Norf, the EPR characteristic signal from $\bullet O_2^-$ is weak and can be ignored compared with that from •OH of Norf. For PMB and SMT, no the characteristic peaks of $\bullet O_2^-$ are observed in their EPR spectra, suggesting their poor ability of producing $\bullet O_2^-$ under light irradiation.

Singlet Oxygen Sensor Green (SOSG) reagent is highly selective for $^1O_2$ and does not show any appreciable response to •OH or $\bullet O_2^-$ under light irradiation.[41] As shown in Figure 5d, the fluorescence signal of SOSG with SMT under light irradiation is remarkably higher than that of without light irradiation or SOSG itself, indicating that SMT can produce strong $^1O_2$ under light irradiation. For PMB, Norf, and Teic, the fluorescence intensities of SOSG with them under light irradiation increase slightly as compared with those of without light irradiation, indicating that the three antibiotics possess poor ability to produce $^1O_2$ under light irradiation.

Table 1. Summary of the specific types of ROS produced by antibiotics

	Antibiotics			
ROS	PMB	SMT	Norf	Teic
•OH	+	+	+	-
$•O_2^-$	-	-	-	+
$^1O_2$	-	+	-	-

Note. "+" and "–" indicate the positive and negative activity, respectively.

The specific types of ROS under light irradiation were summarized in Table 1. The results indicate that different types of antibiotics with PDT activity can produce different types of ROS, such as •OH, $•O_2^-$, and $^1O_2$, under light irradiation. We conclude that the antibacterial activity of the light-activated PMB and Norf may mainly be attributed to the •OH production. SMT performs the photodynamic killing of bacteria mainly due to the production of $^1O_2$ and •OH under light irradiation. The generation of $•O_2^-$ under light irradiation is the main factor of photodynamic antibacterial activity of Teic.

In general, the wavelength of the light source used by PDT matches the absorption band of the photosensitizer. However, as shown in Figure S4, the absorption band of all four antibiotics are in the ultraviolet region (<400 nm), which does not match the visible light source used in this work. Although the mechanism by which these antibiotics

produce ROS remains to be understood, the results of the light-activated antibiotics in this study are helpful to understand the photodynamic activity of different antibiotics and develop new efficient antimicrobial antibiotics with their PDT ability.

Conclusions

In summary, we report that the existing antibiotics, such as PMB, SMT, Norf, and Teic, are capable of producing ROS under light irradiation, suggesting that these antibiotics can be directly used as photosensitizer for the photodynamic killing of bacteria. All of these light-excited antibiotics give more than about 3 orders of magnitude of bacterial killing efficiency, comparable to those of light-excited tetracyclines. These antibiotics used in antibacterial PDT have more advantages than the general photosensitizers because of their intrinsic antibacterial properties and the specific targeted binding ability with bacteria. Moreover, although different antibiotics have their own unique antibacterial spectrum, the photodynamic activity of antibiotics endows them broader applications due to their enhanced antibacterial ability. In addition, the visible light source used in the light-activation study is less harmful to bacteria than the ultraviolet or blue light source used in the light-activated tetracyclines. Therefore, this antibiotic-

based PDT method can extend the application of the existing antibiotics and reduce the therapeutic dose of these antibiotics, thereby reducing the emergence of resistance. We are conducting further research to discover more light-activated antibiotics. Due to the large number and variety of antibiotics at present, further screening of antibiotics with photodynamic activity can provide a novel, simple, and efficient way for treatment of bacterial infections, and even cancers.

Experimental Section

Materials and reagents.

Ampicillin, Kanamycin sulfate, Polymyxin, Sulfamethizol, and Norfloxacin were purchased from Sigma. Teicoplanin was purchased from Selleck. Aminopyridinium iodide (PI) dye was obtained from Beyotime. Fluorescence dyes 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA), 3′-(p-hydroxyphenyl) fluorescein (HPF), Singlet Oxygen Sensor Green (SOSG), and cell culture plates were purchased from Thermo Fisher. *Escherichia coli* (*E. coli*, BL21) was purchased from Beijing Bio-Med Technology Development Co., Ltd. The kanamycin-resistant *E. coli* BL21 (kana^r *E. coli*) was obtained by transfecting kanamycin resistant plasmids of pET28a-PE66.[42]

Staphylococcus aureus (*S. aureus*) ATCC 6538 was obtained from China General Microbiological Culture Collection Center. Phosphate-buffered saline (PBS) buffer (20×) was purchased from Sangon Biotech (Shanghai, China).

Bacterial culture

First, a kana^r *E. coli* or *S. aureus* picked from a single colony was transferred to 10 mL of the Luria-Bertani (LB) medium with kanamycin (50 μg/mL) for *E. coli* or LB medium for *S. aureus*, and then incubated in the dark at 37 °C and 180 rpm for about 6 h to an optical density of 0.8 at 600 nm (OD_{600} = 0.8). The optical densities at 600 nm of the bacteria were measured using a TU-1901 UV-vis spectrophotometer (Purkinje, China). Afterward, we transferred the defined amount of bacterial solution for centrifugation at 8000 rpm for 3 min and discarded the supernatant. Then, the bacterial solution was washed with PBS buffer (1×) two times and suspended with LB medium or PBS (1×) for the subsequent studies.

Antibacterial activities of antibiotics against bacteria

The bacterial viability of kana^r *E. coli* or *S. aureus* treated with antibiotics under light irradiation was performed as follows. First, the above 0.8 OD bacterial solutions were

diluted 40 times. Next, aliquots of 80 μL bacterial solution were transferred to a 96-well plate at a density of about 1.6×10^5 bacterial counts/well in LB medium. Then, different concentrations of antibiotics were added to the bacteria solution and the final volume of each well was 100 μL with LB medium. Subsequently, the 96-well plates were irradiated by a MVL-210 visible light source equipped with a metal halogen lamp (Mejiro Genossen, Japan) at different light intensity and different time. Afterward, the plates were cultured at 37 ℃ and 180 rpm overnight about 16 h.

Finally, the absorbance of solution at 600 nm was measured with a Microplate Reader SpectraMax M2 (Molecular Devices) and the bacterial viability was calculated according to the following equation: Bacterial viability (%) = [(C-B)／(A-B)] × 100%, where A was the absorbance of the mixture of bacteria without addition of antibiotic as the control, B was the absorbance of the equivalent LB medium solution as background, and C was the absorbance of the mixture of bacteria with different concentration of antibiotics as sample without or with light irradiation.

Antibacterial activity test by colony counting

Aliquots of 800 μL of the above diluted 40 times bacterial solutions were transferred in 48-well plate. Then, four portions of the bacterial solution were added by LB solution or/and antibiotics to the final volume 1 mL, respectively. The four bacterial solutions contained bacteria only, bacteria treated with antibiotic, bacteria treated with light irradiation, and bacteria treated with antibiotic under light irradiation, respectively. The light irradiation was performed with 50 mW/cm^2, 40 min for kana^r *E. coli* and 40 mW/cm^2, 40 min for *S. aureus*. Then, each sample was serially diluted multiple folds with PBS (1×). Subsequently, a 100 μL portion of the dilution bacteria was evenly spread on the solid LB agar plate. The plates were cultured at 37 ℃ for about 18 h. Suitable plates were selected and the number of colony forming units (CFUs) was counted. Plate counting photographs of the bacteria diluted by the same times were taken by a Nikon D90 digital camera.

CLSM and SEM characterization of bacteria treated with light irradiation and/or antibiotic

For CLSM imaging of the bactericidal effect of PMB, in two 1.5 mL centrifuge tubes, 1 mL the above kana^r *E. coli* solutions, which contained bacteria treated with antibiotic

and bacteria treated with antibiotic under light irradiation, were taken, respectively. After incubating for 30 min, the solutions were centrifuged with 8000 rpm for 2 min, and washed with PBS (1×) for two times. For CLSM imaging, the E. coli solutions were stained with 1 μg/mL PI dye for 15 min and then washed with PBS for three times. Finally, the precipitations were suspended with glutaraldehyde (0.5%) for 30 min, and subsequently centrifuged with 8000 rpm for 2 min. After removing the supernate, the E. coli pellets were suspended with 20 μL PBS buffer (1×). Finally, 10 μL *E. coli* suspensions were transferred to a glass slide to be imaged with a FV1000-IX81 confocal laser scanning microscope (Olympus).

For SEM imaging, the bacterial pellets were collected and immediately fixed with glutaraldehyde (0.5%) in PBS buffer at room temperature for 30 min. After centrifugation and re-suspension, 5 μL of bacterial suspensions were added to a clean silicon slice followed by naturally drying in the air. Then, the specimens were fixed with 0.1% glutaraldehyde in PBS buffer for 1 h and 0.5% glutaraldehyde overnight, respectively. After washing with sterile water for two times, the specimens were dehydrated by the addition of ethanol in a graded series for 6 min, respectively. Finally, the specimens

were sprayed with platinum for SEM images, which were generated in the JSM-7500F scanning electron microscope (JEOL).

Detection of ROS in solution by DCFDA, HPF, and SOSG

First, the total ROS generated from antibiotics PMB, SMT, Norf, AMP, Kana, and Teic was detected by a ROS-sensitive probe, DCFDA, under light irradiation. Briefly, 100 μL antibiotic (1mg/mL) and 200 μL activated DCFDA (40 μM) solution were added to 700 μL PBS buffer (25 mM, pH=7.4). After the mixtures were irradiated with light irradiation (40 mW/cm^2) for different time intervals, the fluorescence spectra of DCF solution were recorded on an F-7000 fluorometer (Hitachi, Japan) from 505 to 650 nm with the excitation wavelength of 480 nm.

For intracellular detection of ROS, a ROS assay kit (Beyotime) was selected and followed by the manufacturer's procedures. Specifically, in two 1.5 mL centrifuge tubes, 80 μL kana^r *E. coli* (0.8 OD) solutions were diluted with 919 μL LB medium and then stained with 1 μL DCFDA (10 mM). After incubating at 37 ℃, 180 rpm for 20 min, the solutions were centrifuged with 8000 rpm for 3 min. By discarding the supernates, the precipitations were suspended with 1 mL LB medium without and with 0.64 μM PMB,

respectively. Then, the bacterial solutions were transferred to a 48-well plate. Subsequently, the 48-well plates were irradiated by a MVL-210 visible light source equipped with a metal halogen lamp (Mejiro Genossen, Japan) with 40 mW/cm^2 light irradiation for 10 min. Next, the bacterial solutions were transferred to two 1.5 mL centrifuge tubes and centrifuged at 8000 rpm for 3min, respectively. After discarding the supernatants, the precipitations were resuspended with 50 μL PBS buffer (1×). Finally, 10 μL *E. coli* suspensions were transferred to a glass slide to be imaged with a FV1000-IX81 confocal laser scanning microscope (Olympus).

Hydroxyl radical (•OH) was detected by using HPF. Specifically, 100 μL antibiotics (1 mg/mL), including PMB, SMT, Norf, Teic, and 100 μL HPF (25 μM) were added to 800 μL PBS buffer (25 mM, pH=7.4). Then, the mixtures were exposed to light irradiation (40 mW/cm^2) for different time intervals. The fluorescence spectra of HPF solution were recorded on an F-7000 fluorometer (Hitachi, Japan) from 505 to 650 nm with the excitation wavelength of 490 nm.

SOSG reagent was used for special detection of singlet oxygen ($^1O_2$). Briefly, 100 μL SOSG (25 μM) solution and 100 μL antibiotics (1 mg/mL), including PMB, SMT, Norf,

and Teic, were added into PBS buffer (25 mM, pH=7.4) with the final volume of the mixture as 1 mL. The mixtures were irradiated by light irradiation (40 mW/cm^2) for different time intervals. The fluorescence spectra of SOSG solution were measured on F-7000 fluorometer from 500 to 650 nm with the excitation wavelength of 480 nm.

EPR spectra of radical anions under light irradiation

70 μL antibiotic (10 mg/mL in sterile water) was mixed with 70 μL 5,5-Dimethyl-1-pyrroline- n-oxide (DMPO, 100 mM in sterile water) as trapping reagent for detection of •OH. For detection of superoxide radical (•O_2^-), 70 μL antibiotic (10 mg/mL in methanol) was mixed with 70 μL DMPO (100 mM in methanol) as trapping reagent. Then, the mixture was sucked up by capillary with diameter of 0.5 mm. After sealing both sides of capillary by rubber plug, the capillary was put into the resonator with a high-pressure mercury lamp (USH-500SC) irradiation to capture the free radical at 25 ℃ for 2 min. EPR signals of the antibiotics in capillary could be directly acquired on a JES-FA200 ESR Spectrometer.

ASSOCIATED CONTENT

Supporting Information.

The Supporting Information is available free of charge.

Total ROS detection from Kana and AMP by fluorescence dye DCFDA, viability of kanar *E. coli* treated with Kana and AMP, effects of light irradiation time and light intensity on the viability of kana^r *E. coli*, absorption spectra of the antibiotics, IC90 values of the antibiotics for treatment of bacteria.

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Author Contributions

Y.C. and J.Z conceived and designed the research. Q.J., F.E., J.T., and J.Y. performed the experiments. Y.C. and J.Z. analyzed the data and wrote the manuscript. All the authors discussed the results and edited the manuscript.

Notes

The authors declare no competing financial interest.

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BRIEFS

Some existing antibiotics can produce reactive oxygen species (ROS) directly under light irradiation for potentiating bacterial killing.

SYNOPSIS