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Paradoxical potentiation of methylene blue-mediated antimicrobial photodynamic inactivation by sodium azide: Role of ambient oxygen and azide radicals

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Abstract

Sodium azide (NaN_3) is widely employed to quench singlet oxygen during photodynamic therapy (PDT), especially when PDT is used to kill bacteria in suspension. We observed that addition of NaN_3 (100 μM or 10 mM) to gram-positive *Staphylococcus aureus* and gram-negative *Escherichia coli* incubated with methylene blue (MB) and illuminated with red light gave significantly increased bacterial killing (1–3 logs), rather than the expected protection from killing. A different antibacterial photosensitizer, the conjugate between polyethylenimine and chlorin(e6) (PEI-ce6), showed reduced PDT killing (1–2 logs) after addition of 10 mM NaN_3 . Azide (0.5 mM) potentiated bacterial killing by Fenton reagent (hydrogen peroxide and ferrous sulfate) by up to 3 logs, but protected against killing mediated by sodium hypochlorite and hydrogen peroxide (considered to be a chemical source of singlet oxygen). The intermediacy of $\text{N}_3^\bullet$ was confirmed by spin-trapping and electron spin resonance studies in both MB-photosensitized reactions and Fenton reagent with addition of NaN_3 . We found that $\text{N}_3^\bullet$ was formed and bacteria were killed even in the absence of oxygen, suggesting the direct one-electron oxidation of azide anion by photoexcited MB. This observation suggests a possible mechanism to carry out oxygen-independent PDT.

Keywords

Sodium azide; Antimicrobial photodynamic therapy (APDT); Hydroxyl radical; Singlet oxygen; Azidyl radical

Introduction

Bacterial infections have been a constant threat to human health throughout history. There is an ever-increasing incidence of antibiotic resistance that can sometimes involve multiple classes of antibiotics [1] and is spreading around the world not only in hospitals [2] but also in the community [3]. This phenomenon has led several authors to speak of the forthcoming “end of the antibiotic era” [4–8]. Thus, the search for novel, more efficient antibacterial therapies has been a subject of intense and continuing research efforts [9]. Antimicrobial photodynamic therapy (APDT) has been proposed as an alternative treatment for localized infections to which it is hypothesized, bacteria will not easily develop resistance [10]. APDT combines a nontoxic photoactivatable dye, or photosensitizer (PS), in combination with harmless visible light of the correct wavelength to excite the dye to its reactive triplet state [11]. The long-lived triplet PS, via interaction with molecular oxygen and appropriate electron donors or acceptors, is able to produce singlet oxygen and other cytotoxic partially reduced oxygen species, collectively called reactive oxygen species (ROS). Triplet-state PS–oxygen reactions may proceed in two distinctly different manners leading to different types of ROS: The Type 1 mechanism involves electron transfer and leads to free radicals, such as superoxide anion and, via secondary reactions involving hydrogen peroxide, hydroxyl radical ($\text{HO}^\bullet$). The Type 2 mechanism leads to the formation of singlet oxygen ($^1\text{O}_2$), which is not a free radical but like reactive free radicals is able to directly destroy microorganisms. Both Type 1 and Type 2 reactions can occur simultaneously, and the ratio between these processes depends on the type of PS used and critically on the microenvironment of the PS molecule. Moreover the situation is further complicated by the fact that the initially produced $^1\text{O}_2$ can subsequently react with biological substrates (such as unsaturated fatty acids) to produce secondary radicals (such as lipid peroxide radicals). Although some progress has been made over the past few years, the mechanistic details of how APDT affects microbial cells are not fully understood.

In the early 1990s it was realized [12] that gram-negative bacteria were only efficiently killed by PS that had a pronounced cationic charge and it was hypothesized [13] that the cationic dyes were taken up into gram-negative bacteria by the self-promoted uptake pathway first described by Hancock [14]. The short diffusion distance of both $^1\text{O}_2$ and of most radicals produced during PDT explained the increased efficacy when they were produced from dyes located inside microbial cells. In recent times it has become possible to design broad-spectrum PS that are able to effectively kill gram-positive bacteria, gram-negative bacteria, and fungi [15–17].

The outer membrane (OM) of gram-negative bacteria acts as a permeability barrier that is able to exclude macromolecules and hydrophilic substances, thereby being responsible for the intrinsic resistance of these bacteria to antimicrobial compounds [18–20]. Polyethylenimine (PEI), a cationic polymer, has been recognized as a permeabilizer of pathogenic gram-negative bacteria acting by intercalating into the OM [21–23]. Chlorin(e6) (ce6) has been used as a second-generation photosensitizer since it absorbs long-wavelength light and has a high photosensitizing efficacy [24]. In 1997 our laboratory formed the hypothesis and then subsequently demonstrated that by covalently conjugating a suitable PS such as ce6 to either a poly-L-lysine chain [25,26], or to a polyethylenimine chain to form PEI-ce6 conjugate [27], a bacterially targeted PS delivery vehicle could be constructed that would efficiently inactivate both gram-positive and gram-negative species in vitro [28,29] and in vivo [30–32]. PEI-ce6 is considered as a Type 2 PS [33].

Methylene blue (MB) is a phenothiazinium dye with a cationic charge at neutral pH that has been reported to be an effective PS inactivating a wide range of microorganisms after

illumination with red light. It has been reported that photodynamic activity of MB occurred in part, via a Type I mechanism in which $\text{HO}^\bullet$ was produced [33,34].

Several chemical reactions can be used as nonphotochemical means of ROS generation. The combination of sodium hypochlorite and hydrogen peroxide (H_2O_2) can produce $^1\text{O}_2$ [35,36]. It is well known that the Fenton reaction ($\text{H}_2\text{O}_2/\text{Fe}^{2+}$) can produce $\text{HO}^\bullet$ [37]. In this study, we used two different PS: MB (considered under the experimental conditions used to act mostly via Type 1 mechanism) and PEI-ce6 conjugate (Type 2 mechanism) expecting to see quenching of killing by NaN_3 . NaN_3 is well known as a physical quencher of $^1\text{O}_2$ [38] and has been used as an inhibitor of PDT-induced microbial cell killing caused by $^1\text{O}_2$ during PDT [39]. When 10 mM NaN_3 was added to PEI-ce6-mediated PDT, killing was, as expected, reduced for both the gram-positive bacterium *Staphylococcus aureus* and the gram-negative bacterium *Escherichia coli*. However, we found surprising results in that the addition of NaN_3 to MB-mediated PDT did not reduce bactericidal activity, but actually potentiated the PDT effectiveness for both *S. aureus* and *E. coli*. Perhaps even more puzzling was the observation that in the presence of azide the bacterial killing mediated by MB did not require oxygen.

Materials and methods

Chemicals

Methylene blue, sodium azide (NaN_3), ferrous sulfate (FeSO_4), sodium hypochlorite (NaOCl), and 30% hydrogen peroxide (H_2O_2) were purchased from Sigma-Aldrich (St. Louis, MO). The synthesis and molecular characterization of conjugate PEI-ce6 have been previously described in detail. The conjugate PEI-ce6 was employed as 2 mM stock solution in distilled water. MB was dissolved in distilled water to give stock solutions with concentrations of 1 mM. The chemical structures and absorption spectra of the PS are shown in Fig. 1. It can be seen that both dyes were efficiently excited by the 660 $\pm$ 15-nm filtered broadband light source. All PS stock solutions were stored at 4 °C in a refrigerator in the dark for no more than 2 weeks. Other chemicals such as NaN_3 , FeSO_4 , NaOCl , and H_2O_2 were prepared in distilled water immediately before experiments.

Bacterial strain and culture conditions

Gram-positive bacterium *Staphylococcus aureus* 8325-4 and gram-negative bacterium *Escherichia coli* K12 (ATCC, Manassass, VA) were used in this study. The bacteria were routinely grown in brain–heart infusion (BHI) broth (Fischer Scientific) with aeration in an orbital shaking incubator at 130 rpm at 37 °C overnight to stationary phase. An aliquot of this suspension was then refreshed in fresh BHI to mid-log phase. Cell numbers were estimated by measuring the optical density [OD] at 600-nm (OD of 0.5=10(8) colony forming units (CFU) cells/ml).

Photodynamic inactivation studies

Suspensions of bacteria (10(8) CFU/ml) were incubated in the dark at room temperature for 30 min with various concentrations of MB or conjugate PEI-ce6 with and without NaN_3 in pH 7.4 phosphate-buffered saline (PBS). Centrifugation (3 min, 12,000 rpm) of 1-ml aliquots was used to remove the excess of PS that was not taken up by the microbial cells when experiments required it.

The 1-ml aliquots were transferred to a 24-well plate and illuminated from the top of the plates in the dark at room temperature with a red light source (660 $\pm$ 15 nm band-pass filter, Lumacare, Newport Beach, CA) to deliver fluences ranging from 0 to 32 J/cm² (8 J/cm² for MB, 32 J/cm² for conjugate PEI-ce6) at an irradiance of 100 mW/cm² as measured with a

power meter (Model DMM 199 with 201 standard head; Coherent, Santa Clara, CA). At times during the illumination when the requisite fluences had been delivered, aliquots were taken from each well (the suspensions were thoroughly mixed before sampling to avoid the settlement of cells). Cells treated with MB or conjugate PEI-ce6 with and without NaN_3 in the dark were incubated covered with aluminum foil for the same time as the PDT groups (30 min). The aliquots were serially diluted 10-fold in PBS to give dilutions of 10^{-1} to 10^{-5} times in addition to the original concentration and 10- μl aliquots of each of the dilutions were streaked horizontally on square BHI agar plates as described by Jett et al. Plates were streaked in triplicate and incubated for 12–18 h at 37 °C in the dark to allow colony formation. Each experiment was performed at least three independent times.

Control groups of cells treated with light alone (no MB and conjugate PEI-ce6 added) showed the same number of CFU as absolute control (data not shown). Survival fractions were routinely expressed as ratios of CFU of microbial cells treated with light and MB or PEI-ce6 conjugate with and without 10 mM NaN_3 (or MB or PEI-ce6 conjugate with and without 10 mM NaN_3 in the absence of light) to CFU of microbes treated with neither.

To remove oxygen, a mixture of cells, PS, and sodium azide (if required) contained in a quartz cuvette (Model 32Q10, Starna Cells Inc., Atascadero, CA), containing a magnetic stirrer and sealed with a rubber septum pierced with a hollow needle, was bubbled with nitrogen for at least 15 min. The quartz cuvette allowed light to be delivered at different fluences without admitting air.

Fenton reagent and NaOCl/ H_2O_2 antimicrobial studies

Suspensions of bacteria (10(8) CFU/ml) were incubated in the dark at room temperature with various concentrations of Fenton reagent ($\text{FeSO}_4/\text{H}_2\text{O}_2$ at equimolar ratios) or NaOCl/ H_2O_2 at 1/4 molar ratios with and without addition of NaN_3 (0.5 mM) in pH 7.4 PBS for 60 min. At the completion of incubation aliquots (100 μl) were taken from each tube to determine CFU. The aliquots were serially diluted as described above.

Electron paramagnetic resonance spin trapping studies

To monitor the formation of radicals in model systems, electron paramagnetic resonance (EPR) spin trapping was employed [40–42], using 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) at a concentration of 50 mM as a spin trap. The formation of DMPO spin adducts with azidyl radicals and superoxide anion was initiated by “in situ” irradiation of appropriate samples, placed in EPR quartz flat cells, with 540–740 nm (70 mW/cm²) light. The light source was a 300-W high-pressure compact arc xenon lamp (Cermax, PE300CE–13FM/Module 300W, Perkin-Elmer) equipped with a water filter, heat reflecting hot mirror, cutoff filter blocking light below 390 nm, and long-pass filter transmitting light above 540 nm. Superoxide anion was detected in samples containing 25 μM MB and 1 mM NADH, using DMSO/ H_2O (80/20% v/v) as solvent. Azidyl radicals were photoinduced after addition to the samples of 25 mM or 10 mM NaN_3 . Hydroxyl radicals and azidyl radicals induced by Fenton reaction were studied under the following conditions: 1 mM H_2O_2 and 0.05 mM FeSO_4 in the presence and absence of 25 mM NaN_3 . FeSO_4 was always added as the last component and the time from FeSO_4 addition to the first EPR scan was on average about 100 s. EPR samples were run using the following instrumental settings: microwave power 10.6 mW, modulation amplitude 0.05 mT, center field 339.0 mT, scan width 8 mT, and scan time 84 s. To establish anaerobic conditions, samples were bubbled with argon for at least 30 min and, without exposure to air, sucked into an EPR flat cell, which was prepurged with argon. Simulations of EPR spectra were performed using the WinSIM (Version 0.98) program. The values of hyperfine splitting constants for the DMPO-OH spin adduct have been taken from [43,44] for DMPO-OOH from [44] and for DMPO- N_3 from [45]. EPR

measurements were carried out using a Bruker EMX-AA EPR spectrometer (Bruker BioSpin, Germany).

Statistics

Values are means of three separate experiments, and bars presented in the graphs are standard errors of the means (SEM). Differences between means were tested for significance by one-way ANOVA and Tukey's post hoc test. *P* values of <0.05 were considered significant.

Results

Azide potentiates MB-PDT killing of *S. aureus* and *E. coli*

We first established that NaN₃ alone incubated with bacteria for 1 h did not begin to show any toxicity until the NaN₃ concentration reached 50 mM, and therefore were assured that 10 mM NaN₃ had no effect on bacterial viability. The effect of added NaN₃ (10 mM) on MB-PDI of *S. aureus* and *E. coli* is shown in Fig. 2A and B. In order to test whether the binding of PS to the bacterial cells was important we carried out the studies both without and with a wash, a centrifugation step after PS incubation and before NaN₃ addition. This would allow us to distinguish between ROS produced from PS in solution and ROS produced in close vicinity to the bacteria from PS bound to the cells.

It is known that PDT killing mediated by MB is significantly reduced by a wash because the binding of MB to the bacterial cells is relatively weak. It is also known [28] that the activity of MB is substantially higher against gram-positive bacteria than against gram-negative bacteria which meant we used a MB concentration between 2 and 10 times higher for *S. aureus* (20–100 μM) than for *E. coli* (200 μM). The lack of killing at 0 J/cm² shows that neither MB in the dark or NaN₃ displayed any appreciable dark toxicity. The addition of NaN₃ potentiated MB-PDT killing of *S. aureus* by about 1 log of killing (10 times more) as shown in Fig. 2A. The addition of NaN₃ potentiated MB-PDT killing of *E. coli* to a similar degree (1 log). These curves were significantly different by one-way ANOVA as indicated by the symbols. Note that when the cells were centrifuged after incubation with MB and then NaN₃ was added the potentiation of PDT killing disappeared. This suggests that the hypothesized action of azide radicals is mainly outside of the bacterial cells, probably because azide anion cannot penetrate the bacterium. The azide radicals can presumably kill bacteria from the outside in both gram-positive and gram-negative species.

Azide inhibits PEI-ce6 PDT killing of *S. aureus* and *E. coli*

Fig. 3A and B display the survival fraction curves obtained with 1 μM PEI-ce6 conjugate for *S. aureus* and 10 μM PEI-ce6 conjugate for *E. coli* with and without 10 mM NaN₃ and with and without a wash. There were no differences between a wash and no wash in the case of PEI-ce6 conjugate because PEI-ce6 conjugate bound strongly to the bacteria. The addition of NaN₃ inhibited the PEI-ce6-mediated PDT killing of *S. aureus* completely (6 log reduction) in the case of a wash and by 2 logs in the case of no wash (Fig. 3A). In the case of *E. coli* the inhibition of killing by NaN₃ was almost complete (4–5 logs) and was similar for wash and no wash. The reason for the incomplete inhibition in the case of *S. aureus* with no wash is unclear. It is possible that PEI-ce6 conjugate might produce very small amounts of HO• during PDT. The N₃• radicals produced from HO• may be better able to kill gram-positive *S. aureus* than gram-negative bacterium *E. coli*. When PEI-ce6 conjugate is in solution (no wash), it may act as a slightly more Type 1 PS, while if it is bound to bacterial cells (wash) it may act as a more Type 2 PS. Since it is likely that the PEI-ce6 conjugate binds more strongly to the more negatively charged gram-negative cells than it does to the less anionic gram-positive cells, then in the latter case there would be expected to be more

PS in solution and more $\text{HO}^\bullet$ generated. This observation suggests that the microenvironment is an important factor in the mechanism of APDT.

Azide potentiation of killing of *S. aureus* and *E. coli* by Fenton reagent

We used Fenton reagent (a mixture of H_2O_2 and Fe_2SO_4), accepted to be a chemical reaction that produces $\text{HO}^\bullet$ to carry out bacterial killing. We also carried out the controls of H_2O_2 alone and Fe_2SO_4 alone) as there has been reported effects of these compounds alone on cell viability and that extracellular iron could protect against H_2O_2 toxicity [46]. The results with and without addition of 0.5 mM NaN_3 are shown in Fig. 4A and B. We could not use the same concentration (10 mM NaN_3) as we used in the PDT studies as solid precipitation (possibly ferrous azide) occurred. Interestingly *S. aureus* needed a higher concentration of Fenton reagent to produce equivalent killing than *E. coli*. NaN_3 potentiated Fenton reagent-mediated bacterial killing by 2–3 logs of both *S. aureus* (Fig. 4A) and of *E. coli* (Fig. 4B).

Azide inhibition of killing of *S. aureus* and *E. coli* by $\text{H}_2\text{O}_2/\text{NaOCl}$

We used a chemical reaction that has been reported to produce $^1\text{O}_2$ [35,36]. The results are shown in Fig. 5A and B. NaN_3 (0.5 mM) inhibited NaOCl and H_2O_2 -mediated killing of both *S. aureus* (Fig. 5A) and of *E. coli* (Fig. 5B) by about 3 logs.

Azide allows MB-PDT bacterial killing in the absence of oxygen

Our hypothesis to explain the potentiation of antimicrobial photoinactivation by azide was that hydroxyl radicals formed from photoexcited MB, an electron donor and oxygen, allowed electron transfer from azide anion to produce azide radical and hydroxide anions. However, this hypothesis was called into question by ESR studies (see later) and an alternative hypothesis that involved direct oxidation of azide anion by photoexcited MB was considered. To test this hypothesis we repeated the bacterial photoinactivation studies in the absence of oxygen. Fig. 6 shows the results. Fig. 6A shows that when MB at 100 μM was used to photoinactivate *S. aureus* in the presence of oxygen, the addition of 100 μM azide gave about 1 log of additional killing at all fluences in agreement with previous results. When oxygen was removed by bubbling with nitrogen, the PDT killing by MB and red light was completely abrogated (Fig. 6B); however, when 100 μM azide was added to MB PDT in the absence of oxygen, robust killing (6 logs) of bacteria was maintained with only modest reduction from the killing obtained in the presence of oxygen. Figs. 6C and 6D show the analogous experiments conducted with *E. coli* using MB at 200 μM . Addition of 100 μM azide to the PDT in the presence of oxygen gave about 3 logs of additional killing at 8 J/cm² (Fig. 6C), while when the experiment was repeated in the absence of oxygen the killing by MB-PDT alone almost disappeared, but 6 logs of bacterial killing were obtained in the presence of azide (Fig. 6D).

Electron spin resonance spin-trapping studies

Irradiation of EPR samples containing 25 μM MB and 1 mM NADH in 80% DMSO with orange light induces the formation of a spin adduct (Fig. 7A) with EPR spectrum that is virtually identical with the simulated EPR spectrum (Fig. 7C) of the DMPO spin adduct with superoxide [42], assuming the following hyperfine splittings: $a_{\text{N}}=1.31$ mT, $\alpha_{\text{H}}^\beta=1.05$ mT, and $a_{\text{H}}^\gamma=0.15$ mT. It must be stressed that the hyperfine splitting parameters of the detected spin adducts depend on the solvent used; nevertheless, the indicated-above values are consistent with those reported by other authors [40]. Fig. 7B shows EPR spectrum of the sample that contained in 25 mM NaN_3 . Although an additional weak EPR signal might be present, the dominant feature is the EPR spectrum of the DMPO-OOH spin adduct as in Fig. 7A. The kinetics of the accumulation of the DMPO-OOH signal with irradiation time for the two

samples is shown in 7D. Interestingly, while the initial rate of DMPO-OOH spin adduct generation is higher for the control sample than for the sodium azide containing sample, it strongly decreases with irradiation time (Fig. 7A and B). Under the experimental conditions employed, azide efficiently quenches almost all singlet oxygen that could in principle be generated by the excited MB molecules, indicating that the mechanism of photoformation of superoxide is independent of singlet oxygen. Fig. 8B shows the EPR spectrum of a spin adduct obtained upon irradiation of a sample containing 25 μM MB and 25 mM NaN_3 . Again Fig. 8A shows the background signal of a complete sample in the dark, and Fig. 8C is the computer simulation of the DMPO- N_3 adduct, assuming the following hyperfine splittings: $a_{\text{N}}^{\text{NO}}=1.44$ mT, $a_{\text{H}}^{\beta}=1.44$ mT, and $a_{\text{N}}^{\beta}=0.31$ mT. Fig. 8D shows the kinetics of appearance of the azidyl radical signal. When a complete sample, but without oxygen, was irradiated with active light, an identical EPR signal of the DMPO- N_3 spin adduct appeared, even though its accumulation kinetics was somewhat slower than in the presence of oxygen (Fig. 10 B).

Fig. 9 shows the EPR signals of DMPO spin adducts obtained using Fenton reagent in the absence (Fig. 9A) and presence (Fig. 9C) of NaN_3 . As can be seen the signal in the sample without azide consists of four equally spaced hyperfine lines with the relative intensities 1:2:2:1, with the following hyperfine splittings: $a_{\text{N}}^{\text{NO}}=1.49$ mT and $a_{\text{H}}^{\beta}=1.49$ mT. The signal is very characteristic for DMPO spin adduct with OH radicals [40,42]. Computer simulation of the spin-adduct signal (Fig. 9B) fully confirms this assignment. On the other hand the EPR signal detected in the sample that contained azide (Fig. 9C) can be identified as DMPO- N_3 (compare EPR spectra in Fig. 8). This similarity provides additional evidence of the involvement of $\text{N}_3^{\bullet}$ in the photochemical systems discussed in this work. Fig. 10

Discussion

We have shown that NaN_3 was able to potentiate bacterial killing mediated by MB-photosensitized processes, while inhibiting (as expected) the bacterial killing by PEI-ce6-mediated PDT. NaN_3 potentiated bacterial killing mediated by Fenton reagent while it inhibited bacterial killing by a chemical source of $^1\text{O}_2$ (combination of sodium hypochlorite and H_2O_2). The potentiating effect of azide on bacterial killing induced by Fenton reagent could be explained by the well-known one-electron oxidation of N_3^- by hydroxyl radicals to azidyl radicals:

The reaction occurs at a near-diffusion-controlled rate, with the corresponding second-order rate constant being $1.2 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ [47]. We initially considered that $\text{HO}^{\bullet}$ radical produced from photoactivated MB by the Type I photochemical pathway could form azidyl radicals via reaction (1). In order to prove the intermediacy of $\text{N}_3^{\bullet}$, we used electron spin resonance–spin trapping employing DMPO to trap both $\text{N}_3^{\bullet}$ and $\text{HO}^{\bullet}$ to demonstrate that both radicals were produced from Fenton reagent and that the photoexcited MB is able to oxidize azide to $\text{N}_3^{\bullet}$. It is well accepted that after a PS reaches the excited singlet state, it relaxes to the longer-lived triplet state. From this point, the triplet-state PS may interact with O_2 , forming ROS including radicals particularly $\text{O}_2^{\bullet-}$ and $^1\text{O}_2$ (not a radical). It may also interact with electron donors generating different free radicals. We have also confirmed that in the presence of a biological electron donor, such as NADH, photoexcitation of MB leads to Type I reaction with efficient formation of superoxide anion and, presumably, hydrogen peroxide. Such an indirect photosensitized generation of superoxide anion has been demonstrated in other studies using other photosensitizers [41,48,49]. Importantly, the

formation of superoxide anion and, subsequently, hydrogen peroxide is facilitated by the reducing properties of the NAD radical generated in reaction (2):

where $^3\text{MB}^+$ and $^3\text{MBH}^{2+}$ are the unprotonated and protonated form of the lowest triplet excited state of methylene blue, $\text{MB}^\bullet$ and $\text{MBH}^{\bullet+}$ are the unprotonated and protonated one-electron reduction products of the photosensitizer, and $\text{NAD}^\bullet$ is the radical form of NADH.

The one-electron reduction potential of the couple $\text{NAD}^+/\text{NAD}^\bullet$ is -0.94 V [50], making the reduction of molecular oxygen to superoxide anion very efficient. Indeed, the corresponding bimolecular rate constant of reaction (3) has been determined to be $2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ [51].

Of course, it cannot be ruled out that under such conditions, adventitious redox-active metal ions, such as iron or copper, could catalyze free radical decomposition of hydrogen peroxide (formed via dismutation of superoxide anion) and generate hydroxyl radicals. Although in the presence of high concentrations of azide, a significant percentage of hydroxyl radicals will then be converted to azidyl radicals, it is unclear whether in the biological systems studied, reactions photosensitized by MB could yield substantial fluxes of hydroxyl radicals, responsible for the observed toxicity. Our initial hypothesis needed to be modified when we discovered that both formation of azide radicals and bacterial killing could be observed when MB was illuminated in the presence of azide but in the absence of oxygen. Moreover, our spin-trapping experiments demonstrated that the azidyl radical could also be generated by photoexcited MB without oxygen. The proposed reaction, responsible for photosensitized generation of azide radicals, is given below:

Although the $\text{p}K_a$ for $^3\text{MBH}^{2+}$ is 7.2 indicating that, under the experimental conditions used, both the protonated and the unprotonated forms of the methylene blue triplet excited state exist, we consider only the protonated form. This is because the one-electron reduction potential of $^3\text{MBH}^{2+}/\text{MBH}^{\bullet+}$ being 1.33 V is more favorable for oxidation of azide than that of ($^3\text{MB}^+/\text{MB}^\bullet$), which is 1.21 V [52]. If the pH of a bacterial system subjected to PDT were lower, it would favor photoformation of $^3\text{MBH}^{2+}$ and facilitate one-electron oxidation of azide.

Due to the presence of radical species during early evolution and the leakage of radicals during normal cellular respiration, all forms of life have developed efficient mechanisms for quenching radical-based ROS. Superoxide radical anions ($\text{O}_2^{\bullet-}$) are quenched by manganese and copper–zinc versions of superoxide dismutase; H_2O_2 is broken down by catalase to O_2 and H_2O . Although there are no specific biological scavengers of $\text{HO}^\bullet$, this radical interacts very efficiently with many compounds normally present in the cytoplasm or extracellular environment, perhaps the most abundant of which are glutathione and ascorbic acid. Even though certain carotenoids quench singlet oxygen very rapidly, with diffusion-controlled corresponding rate constants, these pigments are not normal constituents of most cells. Therefore it is fair to state that there are no ubiquitous cellular mechanisms capable of quenching $^1\text{O}_2$. Because cells are incapable of protecting themselves against $^1\text{O}_2$, and because the effective radius of damaging action of singlet oxygen in cells might be significantly larger than that of hydroxyl radicals, many PDT researchers have asserted

that $^1\text{O}_2$ is the primary mediator of photodynamic damage in living systems; consequently, resistance mechanisms of malignant tissues and pathogens should be practically nonexistent.

One of the classical procedures to inhibit photodynamic damage is the addition of NaN_3 while performing PDT, particularly used in the case of antimicrobial PDT. Tavares et al. [39] demonstrated that NaN_3 strongly inhibited the bactericidal PDT effects of porphyrin-based PS. Carvalho et al. [53] showed that NaN_3 was capable of reducing the antifungal effects of PDT performed with MB and another phenothiazinium-based PS toluidine blue O. Maisch and colleagues [54] used addition of NaN_3 to characterize porphyrin-chromophore-based PS as Type II PS, demonstrating that coincubation with NaN_3 reduced killing of prokaryotic and eukaryotic cells and that radical scavengers had no effect on PDT killing. Finally, Bose and Dube [55] demonstrated that addition of N_3^- reduced the PDT-catalyzed oxidation of tryptophan.

In agreement with the aforementioned papers, our study demonstrated that NaN_3 is capable of inhibiting the PDT-mediated bacterial killing by the Type II PS, PEI-ce6, by 3–6 logs presumably by quenching $^1\text{O}_2$. However, contrary to these works, we demonstrated that NaN_3 added in the presence of PDT with the Type I PS MB *enhanced* photoantibacterial activity by 1–3 logs. These findings strongly suggest that $^1\text{O}_2$ has little role in the eradication of bacteria by MB-PDT and that other ROS are chiefly involved.

To test this hypothesis, we exposed bacterial cells to $\text{NaOCl}/\text{H}_2\text{O}_2$ —a chemical means of generating $^1\text{O}_2$, routinely employed by neutrophils in the destruction of foreign bodies—and the Fenton reaction, $\text{Fe}^{2+}/\text{H}_2\text{O}_2$, a chemical reaction to generate $\text{HO}^\bullet$. In accordance with the observation that N_3^- quenches $^1\text{O}_2$, the bactericidal activity of the $\text{NaOCl}/\text{H}_2\text{O}_2$ system was reduced by NaN_3 , and, as we predicted based in our hypothesis, NaN_3 potentiated the Fenton reaction by up to 3 logs when used at a concentration of 0.5 mM.

Halide anions such as iodide are capable of potentiating $\text{HO}^\bullet$ -mediated damage, consistent with findings of Sugar et al. [56], who showed that the Fenton system's fungicidal activity is potentiated by the presence of iodide. This is presumably due to the formation of longer-lived iodide radical species. N_3^- is considered a pseudohalide species, whereby the triatomic anion N_3^- collectively behaves like a halide anion. Accordingly, we hypothesized that the observed potentiation of MB-PDT and Fenton reaction was caused by the formation of a $\text{N}_3^\bullet$ radical. Using ESR with DMPO spin trapping, we demonstrated that the azidyl radical ($\text{N}_3^\bullet$) is formed both by Fenton reaction and by MB photosensitized oxidation of azide. Our demonstration that $\text{N}_3^\bullet$ is formed when NaN_3 is added to MB-PDT may explain the observed enhancement of bacterial cytotoxicity: The rate constants of the oxidation reactions between $\text{N}_3^\bullet$ and various organic substrates are usually higher than those of reactions containing other radical species [57,58]. Moreover, N_3^- in the ground state may directly quench the triplet-PS. Killing may also be further enhanced by the known N_3^- inhibition of metalloenzymes, including peroxidases [59].

The lower reactivity of $\text{N}_3^\bullet$ (redox potential 1.33 V) vs $\text{HO}^\bullet$ (redox potential near 2 V [60]) may mean that $\text{N}_3^\bullet$ radicals survive long enough to react better with the fluorescence probe than the extremely short-lived $\text{HO}^\bullet$. Interestingly NaN_3 significantly potentiated the Fenton-mediated killing of both bacterial classes (and by approximately the same amount). If we are correct in presuming that $\text{N}_3^\bullet$ are formed, then these less reactive radicals may also survive long enough to penetrate deeper into microbial cells compared with the more reactive $\text{HO}^\bullet$ that may all expend themselves at the outer surface of the bacterial cells. There have been a few papers that discuss the possibility of azide radical being formed from $\text{HO}^\bullet$ and NaN_3

and potentiating various kinds of biological damage. Roberts [61] found that addition of 10 mM azide potentiated hemolysis by ^{60}Co ionizing radiation and, since this potentiation was prevented by $\text{HO}^\bullet$ scavengers, attributed the observation to production of $\text{N}_3^\bullet$ from $\text{HO}^\bullet$. Land and Prutz [62] found that addition of 10 mM azide potentiated the inactivation of enzymes by pulse radiolysis and attributed the finding to production of $\text{N}_3^\bullet$ from $\text{HO}^\bullet$. Ali et al. [63] reported that the hemolysis of human red blood cells by a photoactivated (cool fluorescent light) riboflavin–Cu(II) system was enhanced by addition of 150 μM azide. They attributed their observation to inhibition of the photodegradation of riboflavin, but commented that “as the riboflavin–Cu(II) system has been shown to produce $\text{HO}^\bullet$ the intermediacy of $\text{N}_3^\bullet$ could not be ruled out.”

The results of this study have several implications for PDT research. First, a long-sought goal in the PDT community, namely oxygen-independent PDT may, at least partly, have been realized. The fact that sodium azide strongly potentiates killing in the absence of oxygen suggests that other less toxic anions such as iodide anion may also do this. There are infections caused by anaerobic bacteria that have very low oxygen tensions where the possibility to still be able to do PDT is attractive. Studies of other PS will be necessary to see which structural features lead to potentiation of killing by azide, and which features to inhibition of killing. The mechanism of how the excited state of MB can remove electrons from an anion such as azide will have to be elucidated. Is the radical cation of MB also formed in the electron transfer reaction and if so what happens to it?

In a broader sense, this study opens an avenue to explore other chemical means of PDT potentiation, perhaps by incorporation of other pseudohalide salts or by the formation of larger, longer-lived radical species.

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Fig. 1. Chemical structures and absorption spectra. (A) MB; (B) PEI-ce6; (C) absorption spectra recorded at 10 μ M in H₂O together with emission spectra of the 660 $\pm$ 15-nm red light source. (B) PEI-ce6 spectrum at 10 μ M in water. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Effect of NaN_3 on MB-mediated PDT of bacteria. Bacteria (10^8 cells/ml) were incubated with stated concentrations of MB for 30 min followed or not by a centrifugation step (wash) followed or not by addition of NaN_3 (10 mM) and illumination with up to 8 J/cm^2 of 660-nm light. (A) *S. aureus* and 20 μM MB. (B) *E. coli* and 200 μM MB.

Fig. 3.

Effect of NaN_3 on PEI-ce6-mediated PDT of bacteria. Bacteria (10^8 cells/ml) were incubated with stated concentrations of PEI-ce6 for 30 min followed or not by a centrifugation step (wash) followed or not by addition of NaN_3 (10 mM) and illumination with up to 32 J/cm^2 of 660-nm light. (A) *S. aureus* and $1 \mu\text{M}$ ce6 eq PEI-ce6. (B) *E. coli* and $10 \mu\text{M}$ ce6 eq PEI-ce6.

Fig. 4.

Effect of NaN₃ on Fenton reagent killing of bacteria. Bacteria (10⁸ cells/ml) were incubated with stated equimolar concentrations of H₂O₂ and FeSO₄ with and without NaN₃ (0.5 mM) for 1 h. Controls were also carried out with H₂O₂ alone and FeSO₄ alone. (A) *S. aureus*. (B) *E. coli*.

Fig. 5. Effect of NaN₃ on NaOCl/H₂O₂ killing of bacteria. Bacteria (10⁸) cells/ml were incubated with stated equimolar concentrations of H₂O₂ and NaOCl with and without NaN₃ (0.5 mM) for 1 h. (A) *S. aureus*. (B) *E. coli*.

Fig. 6.

Bacteria (10^8 cells/ml) were incubated with stated concentrations of MB for 30 min and then by addition or not of NaN_3 (100 μM), followed by removal or not of oxygen by bubbling with nitrogen, and illumination with up to 8 J/cm^2 of 660-nm light. (A) *S. aureus* and 100 μM MB in oxygen; (B) *S. aureus* and 100 μM MB in nitrogen. (C) *E. coli* and 200 μM MB in oxygen; (D) *E. coli* and 200 μM MB in nitrogen.

Fig. 7. MB-photosensitized formation of DMPO-OOH spin adducts. Samples contained 25 μM MB, 1 mM NADH, and 80% DMSO. (A) Control without NaN_3 ; (B) with 25 mM NaN_3 (spectra in A and B recorded after irradiation of samples in resonant cavity for 360s with 540–740 nm light); (C) simulation of DMPO-OOH spin adduct; (D) kinetics of accumulation of the spin adduct during sample irradiation; filled circles, control sample without azide; empty squares with 25 mM azide.

Fig. 8. MB-photosensitized formation of DMPO-N₃ spin adducts. Samples containing 25 μ M MB, 25 mM NaN₃, and 50 mM DMPO. (A) Dark control; (B) EPR signal after irradiation of the sample for 270 s with long-wavelength light (540–740 nm); (C) simulation of the EPR signal of DMPO-N₃ spin adduct; (D) kinetics of accumulation of DMPO-N₃ during sample irradiation.

Fig. 9. Formation of DMPO spin adducts from Fenton reagent with and without NaN_3 . EPR signals of spin adducts formed chemically in samples containing: (A) 1 mM H_2O_2 , 0.05 mM FeSO_4 , 50 mM DMPO; (B) simulation of DMPO-OOH spin adduct, (C) 1 mM H_2O_2 , 0.05 mM FeSO_4 , 50 mM DMPO, 25 mM NaN_3 .

Fig. 10. MB-photosensitized formation of DMPO spin adducts in an oxygen-free system. Argon bubbled samples contained 50 mM DMPO, 25 μ M MB and were irradiated for 360 s with 542–742 nm (70 mW/cm^2) light. (A) Control without NaN_3 ; (B) with 10 mM NaN_3 .