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Original Contribution

Thiocyanate potentiates antimicrobial photodynamic therapy: *In situ* generation of the sulfur trioxide radical anion by singlet oxygen

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ABSTRACT

Antimicrobial photodynamic therapy (PDT) is used for the eradication of pathogenic microbial cells and involves the light excitation of dyes in the presence of O_2, yielding reactive oxygen species including the hydroxyl radical ($^{\bullet}OH$) and singlet oxygen ($^1O_2$). In order to chemically enhance PDT by the formation of longer-lived radical species, we asked whether thiocyanate (SCN^-) could potentiate the methylene blue (MB) and light-mediated killing of the gram-positive *Staphylococcus aureus* and the gram-negative *Escherichia coli*. SCN^- enhanced PDT (10 μM MB, 5 J/cm^2 660 nm *hν*) killing in a concentration-dependent manner of *S. aureus* by 2.5 $\log_{10}$ to a maximum of 4.2 $\log_{10}$ at 10 mM ($P<0.001$) and increased killing of *E. coli* by 3.6 $\log_{10}$ to a maximum of 5.0 $\log_{10}$ at 10 mM ($P<0.01$). We determined that SCN^- rapidly depleted O_2 from an irradiated MB system, reacting exclusively with $^1O_2$, without quenching the MB excited triplet state. SCN^- reacted with $^1O_2$, producing a sulfur trioxide radical anion (a sulfur-centered radical demonstrated by EPR spin trapping). We found that MB-PDT of SCN^- in solution produced both sulfite and cyanide anions, and that addition of each of these salts separately enhanced MB-PDT killing of bacteria. We were unable to detect EPR signals of $^{\bullet}OH$, which, together with kinetic data, strongly suggests that MB, known to produce $^{\bullet}OH$ and $^1O_2$, may, under the conditions used, preferentially form $^1O_2$.

Introduction

The increase in disease caused by antimicrobial-resistant microorganisms is a major medical concern of the twenty-first century [1,2]. In the United States, antimicrobial-resistant infections cost an excess of $55 billion and result in a collective 8 million additional hospital days annually [3]. In an effort to reduce morbidity, mortality, and economic losses associated with resistant pathogens, there is currently a global search for alternative antibacterial techniques that are able not only to kill antimicrobial-resistant isolates but also do not cause resistance to said alternative therapeutics.

Photodynamic therapy (PDT) was discovered in 1900 and has since emerged for the treatment of neoplastic conditions [4]. Because PDT can destroy antibiotic-resistant bacteria and selectively kill pathogens over host cells, PDT is considered a promising treatment for localized infections [5]. PDT involves the visible light excitation of a photosensitizer (PS) in the presence of O_2. When light excites a PS, the PS reaches an excited electronic singlet state and may convert to a long-lived excited triplet state which reacts with ground (triplet) state O_2 in two different ways [6]. In the type I photochemical reaction an excited PS molecule transfers an

Abbreviations: DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; MB, methylene blue; mHCTPO, 4-protio-3-carbamoyl-2,2,5,5-tetraperdeuteromethyl-3-pyrrolin-1-yloxy; PB, Prussian Blue; PBS, phosphate-buffered saline; PDT, photodynamic therapy; PS, photosensitizer

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electron to an appropriate acceptor molecule such as ground state O_2. Type I photochemistry forms partially reduced oxygen species, including hydroxyl radicals ($^{\bullet}OH$), superoxide radical anions ($O_2^{\bullet -}$), and hydrogen peroxide (H_2O_2). Alternatively, in the type II photochemical reaction, an energy-transfer reaction occurs between the excited PS and the ground state O_2, forming excited singlet oxygen ($^1O_2$). Both oxygen radicals and $^1O_2$ cause acute oxidative stress, resulting in destruction of target cells. Because of the high reactivity and thus short lifetimes of these reactive oxygen species, oxidative damage by PDT typically occurs in the immediate proximity of PS localization following excitation [7]. In the case of antimicrobial PDT, where dyes localize externally on the surface of bacteria, oxidative damage is limited to the outer portion of the bacterial cell envelope [8].

Recently, we reported the paradoxical potentiation of methylene blue-mediated antimicrobial PDT by azide (N_3^-) [9]—paradoxical as N_3^- physically quenches $^1O_2$, lessening $^1O_2$ killing of bacteria [10]. We concluded that oxygen radicals or the excited PS itself can directly abstract an electron from N_3^-, forming azidyl radicals ($N_3^{\bullet}$) which are less reactive, but more selective, oxidizing agents, because they are longer lived than $^{\bullet}OH$. This longer lifetime may be responsible for enhanced PDT killing as $N_3^{\bullet}$ may diffuse deeply into bacteria and then wreak havoc while the more reactive $^{\bullet}OH$ is consumed at the cell wall. Because N_3^- is a pseudohalide—a collection of atoms that collectively behaves like halides—we subsequently investigated alkali halide salts for potentiation of PDT and observed enhanced antimicrobial activity due to the formation of iodine and bromine radicals (manuscript in preparation). These findings are in accordance with the observation that KBr and KI react with $^{\bullet}OH$ [11].

Pseudohalides besides N_3^-, such as thiocyanate (SCN^-), also function as $^{\bullet}OH$ scavengers [11]. Lassiter et al. augmented lactoferrin-H_2O_2 (a $^{\bullet}OH$ generator) killing of *Streptococcus mutans* via application of SCN^- [12]. SCN^- and $^{\bullet}OH$ react through the following mechanisms:

$$SCN^- + {}^{\bullet}OH \rightarrow SCNOH^{\bullet -} \quad (1)$$

$$SCNOH^{\bullet -} \rightarrow {}^-OH + {}^{\bullet}SCN \quad (2)$$

$$SCN^- + {}^{\bullet}SCN \rightarrow (SCN)_2^{\bullet -} \quad (3)$$

One-electron oxidation of SCN^- by $^{\bullet}OH$ results in the formation of the thiocyanate radical ($^{\bullet}SCN$) [Eqs. (1) and (2)]. $^{\bullet}SCN$ reacts with excess SCN^-, forming the dirhodane radical anion ($(SCN)_2^{\bullet -}$) [Eq. (3)] [13,14]. Even though the SCN^- reaction with radicals provides precedent to analyze the effect of SCN^- on antimicrobial PDT, $^1O_2$, which is also formed, is particularly thiophilic, reacting with sulfur species affording sulfoxides, sulfones, and sulfonic acids [15]. Due to the known reactivity of SCN^- with $^{\bullet}OH$ and sulfur with $^1O_2$, we sought to investigate whether SCN^- enhanced antibacterial PDT and to study the chemical mechanism of any potentiation.

Materials and methods

Chemicals and reagents

Methylene blue (MB), NaSCN, KSCN, $FeSO_4$, $FeCl_3$, KCN, Na_2SO_3, and malachite green were purchased from Sigma-Aldrich (St. Louis, MO). MB stock solutions were prepared in dH_2O and stored at 4 °C in the dark for no more than 24 h prior to use. NaSCN and KSCN solutions were prepared in dH_2O as required immediately before experimentation. The EPR oxygen-sensitive spin probe 4-protio-3-carbamoyl-2,2,5,5-tetraperdeuteromethyl-3-pyrrolin-1-yloxy (mHCTPO) was a generous gift from Prof. H. J. Halpern (University of Chicago, Chicago, IL) [16]. The spin-trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Sigma-Aldrich.

Bacterial strains and culture conditions

Escherichia coli K12 (ATCC 33780) and *Staphylococcus aureus* (NCTC 8325) were chosen as representative gram-negative and gram-positive bacteria, respectively. Bacteria were routinely suspended in brain heart infusion (BHI) broth (Becton, Dickinson, and Company, Franklin Lakes, NJ) and grown overnight, aerobically in a 37 °C shaker incubator (New Brunswick Scientific, Edison, NJ) at 130 rpm. Cells were removed at an optical density at 600 nm (OD_{600}) of 1.0, approximately equivalent to 10^8 colony forming units (CFU) per milliliter as determined by spectrophotometry (Thermo Scientific, Waltham, MA).

Photodynamic inactivation

E. coli and *S. aureus* were harvested at an OD_{600} of 1.0, centrifuged at 12,000 rpm for 3 min (24,148 *g*), and resuspended in phosphate-buffered saline (PBS) to arrest microbial growth. For photodynamic inactivation *E. coli* and *S. aureus* were incubated with MB for 5 min in the dark at final concentrations of 10 μM. PBS suspensions of bacteria exposed to MB were then illuminated with 5 J cm^{-2} of 660 ± 15 nm (100–200 mW) light using a Lumacare LC-122 lamp (Lumacare, Newport Beach, CA) with the appropriate band pass filter [17].

SCN^- combination studies

Cellular toxicity of SCN^- was first determined by preparing bacteria in PBS, as described above and exposing them to KSCN at final concentrations of 10 μM, 100 μM, 1 mM, and 10 mM. PDT SCN^- combination was accomplished by first incubating PBS suspensions of bacteria with SCN^- for 5 min and then exposing these cultures to MB for 5 min. Samples were then irradiated as described above, serially diluted (6 tenfold dilutions), and plated on BHI agar and colonies were counted, following the methods of Jett et al. [18]. PDT killing is reported as the $\log_{10}$ reduction in cell viability relative to control values. Cellular killing is graphed as the mean of three separate experiments. Error bars are standard deviation from the mean.

PDT dependence on oxygen

PDT was performed as described above in the presence of 10 mM KSCN. One-milliliter aliquots were transferred to quartz cuvettes (Model 32Q10, Starna Cells Inc, Atascadero, CA) containing a magnetic stirrer and were sealed with a rubber septum in the dark. Cuvettes allowed O_2-free samples to be irradiated without exposure to ambient air. The septum was pierced with a hollow needle connected to a N_2/Ar line and samples were then bubbled with 75% N_2/25% Ar gas for 10 min. Samples were irradiated with 5 or 10 J cm^{-2} of 660 ± 15 nm *hv* (150 mW/cm^2).

Photosensitized O_2 consumption measurements

Time-dependent changes in O_2 concentration were determined by electron paramagnetic resonance (EPR) oximetry using mHCTPO at concentration 100 μM as an oxygen ($^3O_2$)-sensitive spin probe [16,19]. Samples with and without NaSCN were irradiated in EPR quartz flat cells in the resonant cavity with 540–740 nm (70 mW/cm^2) light derived from a 300 W high pressure compact arc xenon lamp (Cermax, PE300CE–13FM/Module300W, 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. Concentrations of MB and NaSCN were 25 μM and 10 mM, respectively. O_2 consumption measurements were performed in PBS (H_2O and D_2O for