Journal of Photochemistry & Photobiology, B: Biology 165 (2016) 51–57

Contents lists available at ScienceDirect

Journal of Photochemistry & Photobiology, B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Photodynamic inactivation of methicillin-resistant *Staphylococcus aureus* and *Escherichia coli*: A metalloporphyrin comparison

Troy A. Skwor [a,b,*], Stephanie Klemm [a], Hanyu Zhang [c], Brianna Schardt [a], Stephanie Blaszczyk [a], Matthew A. Bork [a]

[a] Rockford University, Department of Chemical and Biological Sciences, 5050 E. State St., Rockford, IL 61108, USA
[b] University of Illinois – Chicago at Rockford, College of Medicine, 1601 Parkview Ave., Rockford, IL 61107, USA
[c] Purdue University, School of mechanical engineering, 585 Purdue Mall, West Lafayette, IN 47907, USA

ARTICLE INFO

Article history:
Received 2 August 2016
Received in revised form 10 October 2016
Accepted 14 October 2016
Available online 15 October 2016

Keywords:
TMPyP
MRSA
E. coli
PDI
Singlet oxygen
Metalloporphyrin

ABSTRACT

Increasing rates of antibiotic resistance coupled with the lack of novel antibiotics threatens proper clinical treatment and jeopardizes their use in prevention. A photodynamic approach appears to be an innovative treatment option, even for multi-drug resistant strains of bacteria. Three components are utilized in photodynamic inactivation: a photosensitizer, light source, and oxygen. Variations in photosensitizers strongly influence microbial binding and bactericidal activity. In this study, four different cationic metalloporphyrins (Cu^{2+}, Fe^{2+}, Pd^{2+}, Zn^{2+}) were compared to the free-base ligand 5,10,15,20-tetrakis(*N*-methylpyridinium-4-yl)porphyrin regarding their electronic properties and generation of reactive oxygen species upon subsequent 405 nm violet-blue irradiation. *Staphylococcus aureus* and *Escherichia coli* were used as representatives of Gram-positive and -negative, respectively, to assess bactericidal effects by the photodynamic process. Bacterial cultures were pre-incubated with porphyrins and exposed to varying doses of 405 nm irradiation (0–30 J/cm^2). Metalloporphyrins containing Cu^{2+} and Fe^{2+} demonstrated minimal effects on viability. Pronounced bactericidal activity was evident with free-base ligand, Zn^{2+}, and Pd^{2+}; though significantly stronger effects were apparent with Pd^{2+}. Photodynamic killing was directly proportional to reactive oxygen species production post-illumination. These data provide new insight into the influence of metal chelation on photosensitizer activity on bactericidal singlet oxygen production. The strong anti-microbial photodynamic action through the use of a portable light-emitting diode over short time intervals (seconds) provides support for its potential use in self-treatment.

1. Introduction

Annually, approximately two million people in the United States acquire bacterial infections that are resistant to antibiotics with 23,000 ensuing deaths [1]. The economic burden of this epidemic claims $20 billion annually in healthcare-associated costs with an additional $35 billion lost due to decreased productivity [2]. Over the last half century, clinical and animal isolates continue to display an alarming upward trend of anti-microbial resistance [3]. This surge in resistant phenotypes can be attributable to the inappropriate use, close proximity of human and animals [4], and improper disposal of antibacterial drugs [5]. *Escherichia coli* are Gram-negative, facultative anaerobic bacteria which inhabit the gastrointestinal tract of warm-blooded mammals. However, *E. coli* is also a well-known etiological agent for gastrointestinal and urinary tract infections in both nosocomial and community-acquired infections. The emergence of extended-spectrum β-lactamases (ESBL) amongst *Enterobacteriaceae* and the first human documented case of resistance to colistin, a last-line treatment for ESBL isolates [6], re-emphasizes the dwindling clinical efficacy of antibiotics.

Beyond Gram-negative bacteria, a similar story prevails amongst Gram-positive bacteria where antibiotic resistant strains of *Staphylococcus aureus* continue to emerge globally [7]. This pathogenic bacterium is responsible for an array of infections including skin, soft tissue, lower respiratory tract, and septicemia. Community and healthcare-acquired methicillin-resistant strains of *Staphylococcus aureus* (MRSA) has dramatically increased [8,9] and claimed over 11,000 lives in the United States in 2011 [1]. Vancomycin, a standard treatment for MRSA, shares an analogous fate where the first resistant case presented in 2002 [10], with succeeding clinical cases of vancomycin-intermediate *Staphylococcus aureus* rising to 7.9% by 2014 [11].

Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*; PDI, photodynamic inactivation; PDT, photodynamic therapy; T4, 5,10,15,20-tetrakis(*N*-methylpyridinium-4-yl)porphyrin; ANOVA, analysis of variance.

* Corresponding author at: Rockford University, Department of Chemical and Biological Sciences, 5050 E. State St., Rockford, IL 61108, USA.
E-mail addresses: tskwor@rockford.edu (T.A. Skwor), stephmarie2626@gmail.com (S. Klemm), zhang220@purdue.edu (H. Zhang), bjschardt@me.com (B. Schardt), stephblaszczyk8@gmail.com (S. Blaszczyk), mbork@rockford.edu (M.A. Bork).

http://dx.doi.org/10.1016/j.jphotobiol.2016.10.016

Since the rate of antibiotic-resistance acquisition is inversely proportional to deficiency in antibiotic discovery, an alternative treatment is imperative. Photodynamic treatment is one example that has shown promise combating numerous bacterial, viral, fungal and protozoan infections [12–16]. Benefits over antibiotics in clinical settings include localized wound application and minimal side effects, resistance, and toxicity [17]. In contrast to antibiotics, sub-inhibitory doses of photodynamic inactivation (PDI) have failed to induce genomic mutations and elevate antibiotic or photodynamic resistance [18,19]. This therapeutic approach utilizes visible light, a photosensitizer, and molecular oxygen to create reactive oxygen species (ROS) resulting in bacterial cell death [20]. An array of chemicals including porphyrins, phthalocyanines, phenothiazines, dyes, chlorines, and acridines have been used as photosensitizers. Specifically, cationic porphyrins like 5,10,15,20-tetrakis(*N*-methylpyridinium-4-yl)porphyrin have shown strong anti-microbial properties to both Gram-positive and -negative bacteria [21,22]; however, modifications of porphyrins could be implemented to enhance inactivation.

The purpose of this study was to investigate antimicrobial effects of PDI using different metalloporphyrins as photosensitizing agents. Cationic porphyrins, as mentioned above, interact with a diverse array of bacterial species. However, subtle changes in the electronic properties of photosensitizers can enhance intersystem crossing thus heightening ROS production and photodynamic damage [23]. Electronic properties can be modified by altering the porphyrin core through metal chelation. This study investigated singlet oxygen production and bactericidal effects against model bacterial pathogens *E. coli* and MRSA using Cu^{2+}, Fe^{2+}, Pd^{2+}, and Zn^{2+} inserted into the tetra-cationic porphyrin. A portable hand-held device with 405 nm light-emitting diodes (LED) was utilized for photosensitizer excitation, thus highlighting its potential as a self-applied treatment for wound infections.

2. Materials and Methods

2.1. Bacterial Culturing and Quantification

E. coli ATCC 11775 and MRSA ATCC 43300 strains were obtained from frozen stocks and cultures grown at 37 °C in tryptic soy broth (TSB). Overnight cultures were centrifuged at 8161 x *g* for 5 min (Eppendorf 5415C, Germany). Cell pellets were washed with 0.85% sterile NaCl (saline) at pH 7.4 and resuspended in 1 ml sterile saline. *E. coli* and MRSA were further diluted in saline to a 0.5 McFarland standard.

2.2. Development of Metalloporphyrins

Metalloporphyrins were synthesized following previously published procedures [24–26]. Briefly, an ion exchange of 5,10,15,20-tetrakis(*N*-methylpyridinium-4-yl)porphyrin tetra(4-toluenesulfonate) yielded the $[H_2(T4)](PF_6)_4$ salt, which was then dissolved in acetonitrile and precipitated with acetone containing tetrabutylammonium nitrate (TBAN). The resulting $[H_2(T4)](NO_3)_4$ was solubilized in distilled water and refluxed overnight with a ten-fold molar excess of a metallic salt: zinc(II) acetate, copper(II) acetate, iron(II) chloride, or $Pd(DMSO)_2(H_2O)_2$ to create various metalloporphyrins. $Pd(DMSO)_2(H_2O)_2$ was synthesized as previously described [27]. Molecular absorption spectroscopy via GENSYS™ 10S UV–Vis Spectrophotometer (Thermo Scientific, MA, USA) was utilized to ensure metal ion insertion. The metalloporphyrin solutions were filtered and isolated by precipitation via addition of KPF_6 in acetonitrile. Ion exchange with TBAN yielded water soluble metalloporphyrins as nitrate salts which were further characterized via absorption spectroscopy to determine concentration and absorption spectra, thus highlighting Soret peaks.

2.3. Determination of Bacterial Binding to Metalloporphyrin

Bacterial suspensions of MRSA and *E. coli* were diluted to a McFarland 0.5 standard with 0.85% sterile saline and incubated with 10 μM of Pd(T4) for 5 min. After incubation, the samples were centrifuged at 8161 x *g*, washed, and subsequently resuspended in 0.85% sterile saline. Absorption spectra of the samples were obtained and normalized. Considering the additive nature of absorbance, quantification of the Pd(T4) interaction with bacteria was estimated from residual Soret peak (at 418 nm) superimposed on the bacterial absorbance.

2.4. Photodynamic Inactivation and Determination of Cell Viability

Preliminary effects of various porphyrins (Cu(T4), Fe(T4), H_2(T4), Pd(T4), and Zn(T4)) against bacterial growth were investigated in 96-well plates. Briefly, 100 μl of MRSA with or without different concentrations of porphyrins were inoculated into wells for 5 min in the dark. Wells were exposed to 405 nm LED (WARP, Quantum Devices, Inc., OH, USA) at 60 mW/cm^2 for 44 s producing 2.5 J/cm^2 and placed on an orbital shaker (MidSci, MO, USA) for 7 h at 200 rpm. Growth was defined by turbidity measurements at 570 nm using an ELx800 Absorbance Microplate Reader (Biotek, VT, USA) post-7 h. After the most effective photosensitizers were identified, bactericidal activity was tested on 2 ml of bacterial culture in 10 × 60 mm plates (Falcon). Porphyrin concentrations or saline alone (negative control) were incubated with bacteria 5 min prior to irradiation (0–30 J/cm^2) in a dark environment. Cell viability was determined by plating 20 μl of various dilutions on tryptic soy agar (TSA) plates and incubating at 37 °C overnight. Colony forming units (CFUs) were counted 18–24 h later. To elucidate the role of reactive oxygen species on bactericidal activity, reduced glutathione (10 mM) was co-administered with porphyrins 5 min preceding irradiation of 0.6 J/cm^2 and 20 J/cm^2 for MRSA and *E. coli* respectively. Bacterial killing was quantified as mentioned above.

2.5. Singlet Oxygen Production

Previous methods were used to measure steady state singlet oxygen production [27]. Briefly, porphyrins were dissolved in D_2O (10 μM) and irradiated with 405 nm LED as excitation source (60 mW). A Fluorolog® 3 Spectrofluorometer (Horiba, NJ, USA) with a liquid nitrogen-cooled indium gallium arsenide detector scanning from 1220 to 1330 nm [28] was utilized to quantify steady state singlet oxygen emission. To assess photodynamic efficiency of singlet oxygen production, spectra were corrected based on the absorbance of photosensitizer solutions (10 μM) at 405 nm using Equation 1: $Em_{cor} = Em_{obs} / (1–10^{(-Abs)})$ where Em_{cor} = corrected emission n; Em_{obs} = observed emission; Abs = absorbance at 405 nm.

2.6. Statistics

Differences between photodynamic inactivation and therapy treatment groups were determined using one-way analysis of variance (ANOVA) with Bonferroni corrections. For bacterial comparisons, the natural log of CFUs were analyzed to meet the assumptions of ANOVA. Statistical analysis was performed using Stata 12 (StataCorp LP, College Station, TX). Outcomes demonstrating $P < 0.05$ were considered statistically significant.

3. Results and Discussion

3.1. Absorption Spectra of Metalloporphyrins and Binding Affinities

For the past seventy years antibiotics have been utilized to treat a multitude of infectious diseases; however, with an absence of new antibiotics in the past thirty years, alternatives are imperative to the clinical field. One alternative is the use of photodynamic treatment encompassing a photosensitizing agent and light. The purview of this study was to assess the influence of incorporating a metal into tetra-cationic porphyrin H_2(T4) on photokilling against MRSA and *E. coli*.