Published in final edited form as:

J Photochem Photobiol B. 2013 December 5; 129: 27–35. doi:10.1016/j.jphotobiol.2013.09.006.

Photo-activated porphyrin in combination with antibiotics: therapies against *Staphylococci*

Sana S. Dastgheyb^a, David M. Eckmann^b, Russell J. Composto^c, and Noreen J. Hickok^d

Sana S. Dastgheyb: Sana.Dastgheyb@Jefferson.edu; David M. Eckmann: David.Eckmann@uphs.upenn.edu; Russell J. Composto: Composto@seas.upenn.edu; Noreen J. Hickok: Noreen.Hickok@jefferson.edu

^aDepartment of Orthopaedic Surgery, Jefferson Medical College, 1015 Walnut St., Suite 501, Philadelphia, PA 19107

^bDepartment of Anesthesiology and Critical Care, University of Pennsylvania, 331 John Morgan Building/6112, 3620 Hamilton Walk, Philadelphia, PA 19104-4215

^cDepartment of Materials Science and Engineering, University of Pennsylvania, 3231 Walnut St., Room 301, Philadelphia, PA 19104

^dDepartment of Orthopaedic Surgery, Thomas Jefferson University, 1015 Walnut St., Suite 501, Philadelphia, PA 19107, 215-955-6979, 215-955-9159 (Fax)

Abstract

Staphylococcal infections have become difficult to treat due to antibiotic insensitivity and resistance. Antimicrobial combination therapies may minimize acquisition of resistance and photodynamic therapy is an attractive candidate for these combinations. In this manuscript, we explore combined use of antibiotics and *meso*-tetra (4-aminophenyl) porphine (TAPP), a cationic porphyrin, for treatment of *Staphylococcus aureus* contamination. We characterize the antimicrobial activity of photoactivated TAPP and show that activity is largely lost in the presence of a radical scavenger. Importantly, TAPP can be reactivated with continued, albeit attenuated, antibacterial activity. We then show that the antimicrobial activity of illuminated TAPP is additive with chloramphenicol and tobramycin for *Staphylococcus aureus* and *Escherichia coli*, and synergistic for MRSA and *Staphylococcus epidermidis*. Chloramphenicol + methylene blue, another photosensitizer, also show additivity against *Staphylococcus aureus*. In contrast, ceftriaxone and vancomycin do not strongly augment the low level effects of TAPP against *S. aureus*. Eukaryotic cells exhibit a dose-dependent toxicity with illuminated TAPP. Our results suggest that even sub-minimum inhibitory concentration levels of photo-activated TAPP could be used to boost the activity of waning antibiotics. This may play an important role in treatments reliant on antibiotic controlled release systems where augmentation with photo-active agents could extend their efficacy.

Keywords

porphyrins; TAPP; antibiotics; *Staphylococci*; *E. coli*; osteoblasts

© 2013 Elsevier B.V. All rights reserved.

Correspondence to: Noreen J. Hickok, Noreen.Hickok@jefferson.edu.

A preliminary report of a portion of this work was presented at the Orthopaedic Research Society Meeting in San Francisco, CA in 2012 and an additional report was presented at the Orthopedic Research Society in San Antonio, TX in 2013.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. INTRODUCTION

The evolving resistance of many bacterial strains makes the exploration of new antimicrobial therapies paramount especially for *Staphylococci* which are prevalent in both deep and cutaneous infection in humans [1]. Among the most successful therapies are combinations of drugs that, by targeting complementary pathways, can treat infection while minimizing acquisition of resistance [2]. The prevalence of *MRSA* (Methicillin-resistant *Staphylococcus aureus*)¹, underlines this need [3]. In the case of cutaneous or subcutaneous infection, these combinations can include topical [4] treatments such as photodynamic therapy with porphyrins [5]. As with classical antibiotics, combinations of porphyrin-based photodynamic therapy with antibiotics may give increased efficacy and have the added advantage of lessening the likelihood of resistance; these combinations are currently underexplored.

Porphyrins are biologically important macrocyclic compounds that are relatively stable due to their extended aromatic structure as well as their ability to complex transition metals [6]. Porphyrins absorb a significant portion of the visible spectrum [7], and in the presence of oxygen produce reactive oxygen species (ROS) that can contribute to cell death [8–11]. Specifically, this production of ROS makes porphyrins useful for cancer treatment [12] as well as for antimicrobial use in light-accessible areas (e.g. cutaneous and subcutaneous areas) [9]. Importantly, porphyrins at antibacterial concentrations generally lack eukaryotic cytotoxicity in the absence of light [7].

Combinations of the photosensitizer methylene blue with several antibiotics can decrease *Burkholderia cepacia* (*B. cepacia*) contamination whereas sequential treatments of *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Staphylococcus aureus* (*S. aureus*) biofilms with the porphyrin *meso*-tetra (N-methyl-4-pyridyl) porphine tetra tosylate (TMP) followed by antibiotics decrease number of biofilm-associated bacteria [13–15]. In the case of *B. cepacia*, effects with TMP were only modest, whereas the sequential treatments of biofilms followed by antibiotics for *P. aeruginosa* and *S. aureus* were efficacious; unfortunately, combinations were not tried in these latter experiments. In this paper, we hypothesize that the porphyrin *meso*-tetra (4-aminophenyl) porphine (C₄₄H₃₄N₈, TAPP), a porphyrin that belongs to the *meso*-substituted cationic porphyrins and whose antimicrobial activity has yet to be characterized, will augment the activity of antibiotics. Since TAPP targets bacterial membranes, we decided to test antibiotics that targeted protein synthesis and thus have a complementary activity to TAPP vs. membrane-active antibiotics which could share sites of action. We test this hypothesis using antibiotics that inhibit membrane integrity (ceftriaxone and vancomycin) or protein synthesis (tobramycin, chloramphenicol) and ask the effects of the combination therapy both at the minimum inhibitory concentration (MIC) and at sub-MIC levels, representative of waning concentrations typical of controlled release systems. We ask if generation of ROS is the cause of the increased activity, and if so, are other photosensitizing agents, such as methylene blue, capable of producing similar combinatory results. Finally we ask if exposure to TAPP causes either resistance to additional TAPP treatments or to other antimicrobials.

¹Abbreviations used: *B. cepacia*: *Burkholderia cepacia*; CFU: colony forming unit; CLSI: Clinical and Laboratory Standards Institute; DDH₂O: distilled, deionized water; DMEM: Dulbecco's modified Eagle Medium; *E. coli*: *Escherichia coli*; EDTA: ethylenediamine tetraacetic acid; FBS: fetal bovine serum; FIC: fractional inhibitory concentration A, B, or I (Index); GSH: glutathione; MIC: minimum inhibitory concentration; *MRSA*: methicillin resistant *Staphylococcus aureus*; *P. aeruginosa*: *Pseudomonas aeruginosa*; PBS: phosphate buffered saline; *S. aureus*: *Staphylococcus aureus*; *S. epidermidis*: *Staphylococcus epidermidis*; ROS: reactive oxygen species; TAPP: *meso*-tetra (4-aminophenyl) porphine; TMP: *meso*-tetra (N-methyl-4-pyridyl) porphine tetra tosylate; TSB: trypticase soy broth.

2. MATERIALS AND METHODS

2.1 Experimental Design

TAPP is a cationic porphyrin (Figure 1a) that upon photoactivation generates ROS (Figure 1b). We measured the photodegradation of TAPP using the experimental setup shown in Figure 1c. We first characterized the photodegradation of TAPP and the effects of TAPP on (1) *S. aureus* (ATCC®25923™), (2) a clinical *MRSA* strain (TJU clinical microbiology laboratories) and (3) *Escherichia coli* (*E. coli* ATCC® 25922™). We asked if 10 and 100 µM TAPP inhibited *S. aureus* growth after 5 h in light (Sylvania 100W full spectrum light) or in the dark and then determined the MIC for TAPP with *S. aureus*, *MRSA*, and *E. coli* using a broth dilution (break-point) assay with 24 h illumination. By serial dilution, plating, and direct counting, the time-dependence of TAPP activity (5 µM, 50 µM) during 1–5 h illumination was measured, as was TAPP (20 µM) activity in the presence of glutathione (0, 5, 10, or 20 mM), a well-known antioxidant [16] that scavenges ROS. We also measured retention of TAPP activity after repeated 5 h light/19 h dark cycles. Bacteria that survived MIC-levels of TAPP were assessed for antibiotic resistance and TAPP resistance using disc diffusion assays. We then tested the ability of TAPP to combine with antibiotics that exhibit inhibition of cell wall synthesis (ceftriaxone, and vancomycin) or protein synthesis (tobramycin and chloramphenicol). *S. aureus* was incubated with these antibiotics at their MIC and at 0.5X MIC, with TAPP at 0.5X MIC and with the combination of antibiotic (0.5X MIC) + TAPP (0.5X MIC) for 5 h illumination. To determine if the effects were additive or synergistic, an array of increasing concentrations of TAPP on the X-axis and antibiotic (chloramphenicol or tobramycin) on the Y-axis was created to form a stepwise gradient (checkerboard assay). Using this assay, the inhibitory concentrations for TAPP and chloramphenicol or tobramycin were determined for *S. aureus*, *Staphylococcus epidermidis* (*S. epidermidis*, ATCC®35984™, ATCC, Manassas, VA), *MRSA*, or *E. coli* after 24 h in the light or the dark. Breakpoints were visually determined and combined effects were calculated using the fractional inhibitory concentration index (FICI) [17]. Also, parallel experiments using identical checkerboard techniques with *S. aureus* were performed with chloramphenicol + methylene blue, which also generates ROS upon exposure to light [18] to substantiate that the production of ROS was critical for the combined effects. Lastly, the toxicity of TAPP towards eukaryotic cells (Saos-2) was assessed under both light and dark conditions.

2.2 Illumination chamber

A humidified, transparent chamber containing the test plate was placed ~12.5 cm below a white light source (100 W, 120 V Sylvania white light) (Figure 1c) with distance adjusted so that the chamber remained at 37°C; controls were covered and incubated in the same chamber.

2.3 Bacteria culture and quantitation

S. aureus ATCC®25923™ (ATCC, Manassas, VA) was grown at 37°C with agitation in trypticase soy broth (TSB, Becton Dickinson & Co., Franklin Lakes, NJ) for 16–18 h. Using a 0.5 McFarland standard (a turbidity standard where a 0.5 McFarland ~1×10⁸ CFU *S. aureus*/mL), *S. aureus* were brought to 10⁸ CFU/mL and diluted in TSB so that ~10⁶ CFU/well (200 µL total volume) were used in each experiment. Similar growth conditions were used to grow *S. epidermidis* (ATCC®35984™, ATCC), *MRSA* (a clinical strain obtained from TJU Clinical Microbiology), or *E. coli* (ATCC®25922™; ATCC). Bacterial viable counts were usually assessed after 0 h and 5 h illumination in white light through serial dilution, plating on TSB Bacto™ Agar (Becton Dickinson & Co) polystyrene Petri dishes (Fisher Scientific), and direct counting.

2.4 MIC determination

Fresh TAPP (1 mM in acidified DDH₂O, pH 5.0, Frontier Scientific, Newark, NJ) or methylene blue (1 mM in DDH₂O, Fisher Scientific, Pittsburgh, PA) was prepared for each set of experiments with dilutions in phosphate buffered saline (PBS). The MIC was determined using Broth microdilution techniques according to the protocol of Clinical and Laboratory Standards Institute (CLSIM31-A2) [19]. Specifically, $\sim 10^6$ CFU/well of bacteria were added to a range of concentrations of TAPP (0–125 μ M) or methylene blue (0–25 μ M) in a 96-well v-bottom plate (final volume 200 μ L, Costar, Corning Life Sciences, Acton, MA). Incubations were performed with and without exposure to light at 37°C, 18 h in a humidified chamber; breakpoints for bacterial survival were determined visually.

2.5 Glutathione treatment

Glutathione (stock: 640 mM in PBS with dilutions in TSB; 98% purity, Fisher Scientific) was added to wells containing 10^6 CFU *S. aureus* bathed in 20 μ M TAPP ($\sim 2\times$ MIC) with final glutathione concentrations of 0 mM, 2.5 mM, 10 mM, 20 mM, and 40 mM). After exposure to light (or no light for control) for 0, 1, or 5 h at 37°C, bacteria were collected, diluted, plated and counted.

2.6 Combinations of photoactivated TAPP and antibiotics

Stock antibiotic solutions were prepared as follows: tobramycin (XGen Pharmaceuticals, Inc., Horsehead, NY): 90 mg/mL in sterile PBS; chloramphenicol (Fluka Biochemika/Sigma-Aldrich, St. Louis, MO): 40 mg/mL in 100% ethanol; ceftriaxone (Sandoz/Novartis, Princeton, NJ): 100 mg/mL in DD H₂O; vancomycin (APP Pharmaceuticals, Lake Zurich, IL): 250 mg/mL in DD H₂O; subsequent dilutions were made in TSB. Initially, sensitivity of *S. aureus* to 0.5X MIC and 5X MIC for tobramycin (4.5 and 45 mg/L), chloramphenicol (2 and 20 mg/L), ceftriaxone (0.5 and 5 mg/L) and vancomycin (0.5 and 5 mg/L) was determined as follows: $\sim 10^6$ CFU of *S. aureus* were added to 0.2 ml TSB containing no antibiotic or antibiotic amounts as listed above. Plates were illuminated for 5 h, with control plates maintained in the same chamber but in the dark. At the end of 5 h, the bacteria in the wells were diluted, plated on TSB-Bacto™ Agar plates, and counted (countable range 30–300 CFU/plate). The combined effect of TAPP with antibiotics was determined using the same experimental procedure where sub-MIC levels of TAPP (5 μ M, 0.5X MIC) were combined with sub-MIC levels (0.5X MIC) of the targeted antibiotic. For these experiments, 4.5 mg/L of tobramycin (data not shown), 2 mg/L chloramphenicol [20], 0.5 mg/L ceftriaxone [21], and 0.5 mg/L vancomycin [20, 22] were tested. Numbers of viable bacteria were determined by counting as above, after exposure to light (or protected from light for the controls).

2.7 Determination of synergy or additivity

To determine additivity or synergy between TAPP and the antibiotics tobramycin or chloramphenicol, a 96 well plate format was used to form a checkerboard on a v-bottom 96-well plate (Costar) [23]. Along each row of the plate, increasing concentrations of tobramycin or of chloramphenicol (0, 0.5, 1, 2, 4, 8, 16, and 32 mg/L) were added. In each column of the plate, increasing concentrations of TAPP were added (0, 0.315, 0.625, 1.25, 2.5, 10, and 20 mg/L for tobramycin; 0, 4, 8, 16, 25, 32, 50 μ M TAPP for chloramphenicol), and the total volume was brought to 200 μ L with TSB. To each well, $\sim 10^6$ CFU of *S. aureus*, *E. coli*, *S. epidermidis*, or of a clinical MRSA strain were added. In parallel, methylene blue was substituted for TAPP with each column containing 0, 0.625, 1.25, 2.5, 5, 10, 20 μ M methylene blue. Each plate was either kept in the dark (control) or photoactivated with white light by illumination for 24 h, 37°C. At the end of 24 h, each well was visually assessed for the presence of a pellet and ranked as either having a pellet or not. Additivity or synergism is then determined using the following equations:

$$FICA = \frac{MIC_{AB}}{MIC_A}$$

$$FICB = \frac{MIC_{BA}}{MIC_B}$$

$$FICI = FICA + FICB$$

IF:

$FICI \leq 0.5$; synergy

$0.5 < FICI \leq 1.0$; additive

$1.0 < FICI \leq 4.0$; indifferent

$4.0 < FICI$; antagonism

2.8 TAPP inactivation assay

Using a 96-well plate, 100 μ L of fresh TSB containing 10 μ M TAPP was exposed to light for 5 h followed by 18–24 h in the dark. This procedure was repeated up to 4 times (5 exposures to light). After each light/dark cycle, 1×10^6 CFU/mL of *S. aureus* were added to the solutions and the combinations were then illuminated (light) or shielded from light (dark) for an additional 5 h; number of viable bacteria were determined by serial dilutions, plating and enumeration. Absorbance (300 – 800 nm) of the TAPP solution was measured for each light/dark cycle using a Tecan Infinite®M1000 PRO plate reader (Männedorf, Germany).

2.9 Eukaryotic cell toxicity assay

Saos-2 (ATCC® HTB-85™; ATCC) cells were grown on 25 mm² tissue culture-treated cover slips (Thermanox, Nunc/Fisher Scientific) in phenol-red free Dulbecco's modified Eagle Medium (DMEM, Mediatech, Cellgro, Manassas, VA) with 10% fetal bovine serum (FBS, heat inactivated; Atlanta Biologicals, Flowery Branch, GA). When cells were near confluence, fresh medium with 0 μ M, 1 μ M, 10 μ M, or 100 μ M TAPP was added and cells were incubated in the dark for 1h followed by incubation with illumination (or no illumination for control) for 5 h, 37°C. Cells were washed with PBS (3x), incubated with 0.2% Trypan Blue (Fisher Scientific) in phenol-red free DMEM +10% FBS, 5 min, washed with PBS (3x), and placed in phenol-red free DMEM +10% FBS for 10–30 min. Cells were imaged with a Nikon EFD3 fluorescent microscope (Melville, NY) under bright field microscopy using Spot™ Advanced (Spot imaging; Sterling Heights, MI) to capture at least three fields of view. Cell toxicity was assessed through manual counting of stained nuclei per field of view.

2.10 Statistical analysis

Statistical analyses were performed using either: 1) A one-way analysis of variance (ANOVA) for the varied treatment conditions compared to untreated controls (alpha was set to 0.05) or 2) A 2-tailed t-Test for comparison of different populations within the same sample. Significance was defined as $p < 0.05$.

3. RESULTS

3.1 Structure and properties of TAPP

TAPP is a metal-complexing cationic porphyrin (Figure 1a), that is activated with visible light in the presence of oxygen to produce singlet oxygen via energy transfer (Figure 1b). The chosen method of excitation is illustrated in Figure 1c where the lamp serves the dual

purpose of illumination and maintaining the 37°C humidified chamber. Non-irradiated TAPP showed strong absorbance in the Soret band (400–500 nm) which is indicative of porphyrins with metal chelates [6] and in the far red region between 650–750 nm. The intensity of the peaks for TAPP diminishes with each light/dark cycle (5 h light; 19 h dark), and by 10 cycles, peak absorbance has decreased by 60% (Figure 1d).

3.2 Photo-activated TAPP is antimicrobial as a function of concentration, time, light cycle, and presence of glutathione

We first determined the antimicrobial properties of TAPP. Photo-activation of both 10 μ M and 100 μ M TAPP caused an ~ 2.5 log decrease in *S. aureus* survival at 5 h; 10 μ M or 100 μ M TAPP in the dark, showed no apparent inhibition of bacterial growth (Figure 2a). After illumination for 24 h, the MIC of TAPP for *S. aureus* was determined to be ~ 10 μ M; for *E. coli*, 60 μ M, and for *MRSA*, 32.5 μ M (data not shown) using the breakpoint assay. In the dark, no apparent toxicity was noted over this same concentration range. Under illumination, 5 μ M (0.5X MIC) TAPP resulted in an ~ 2 log decrease in *S. aureus* growth over the 5 h period. With 50 μ M TAPP (5X MIC), *S. aureus* growth had been significantly decreased at 1 h (~ 2.5 log) of illumination; by 2 h (Figure 2b), bacterial numbers were too few to count; other experiments showed a small residual number of bacteria (data not shown).

We also asked if quenching ROS with the known anti-oxidant glutathione [24] would abrogate the antibacterial effects of illuminated TAPP. Increasing concentrations of glutathione although insufficient to combat the early effects of illumination of 2X MIC (20 μ M) TAPP, attenuated the antibacterial effects at the 5 h time point. Addition of 5, 10 and 20 mM glutathione somewhat abrogated the effects of illuminated TAPP, resulting in only a 2.5–3 log decrease in bacterial number compared to the ~ 5.5 log decrease caused by 20 μ M TAPP alone (Figure 2c). In parallel, we determined the effects of repeated cycles of illumination (Figure 1d) on TAPP activity against *S. aureus*. After 5 h illumination, 10 μ M fresh TAPP resulted in a 4 log decrease in bacterial survival while TAPP that had been illuminated one or two times prior to the incubation with *S. aureus*, consistently resulted in an ~ 2.5 or greater log decrease in bacterial survival. 3X and 5X pre-illuminated TAPP resulted in an ~ 0.5 –1 log decrease in bacterial survival.

In this characterization, we finally asked if the bacteria that survived after incubation with illuminated TAPP retained their normal sensitivity to antibiotics and to TAPP. When bacteria were repeatedly exposed to sub-MIC levels of photo-activated TAPP, they did not show decreased sensitivity (data not shown). When tested for sensitivity to other antibiotics, disc diffusion techniques showed no change in antibiotic sensitivity (data not shown).

3.3 Illuminated TAPP potentiates antimicrobial activities

We now asked if the antimicrobial activity of illuminated TAPP would combine with that of antibiotics to yield enhanced bacterial killing. We tested both sub-MIC (0.5X) and above MIC (5X) concentrations of antibiotics that compromised membrane integrity (ceftriaxone and vancomycin) or antibiotics that inhibit protein synthesis (tobramycin and chloramphenicol) (Figure 3). Viable counts decreased significantly ($p < 0.05$) after treatment with 5X MIC for each antibiotic (measured in CFU ml^{-1}). At 0.5X MIC, ceftriaxone and vancomycin caused little to no decrease in bacterial growth after 5 h (Figure 3a, b). It is worth noting that both tobramycin (4.5 mg/L; Figure 3c) and chloramphenicol (2 mg/L, Figure 3d) caused statistically significant decreases from control, although the effects of 0.5X MIC chloramphenicol (~ 1 log decrease) were much less robust than those of 0.5X MIC tobramycin (~ 2.5 log decrease). In the case of TAPP alone, 0.5X MIC concentrations caused a 1–1.5 log decrease in numbers of viable bacteria when compared to control; this decrease was statistically significant ($p < 0.05$). We asked if the modest effects of sub-MIC

levels of antibiotics could be enhanced by addition of sub-MIC levels of TAPP. When 5 μ M TAPP was combined with 0.5X MIC of the membrane-active antibiotics ceftriaxone (Figure 3a) and vancomycin (Figure 3b), after 5 h, bacterial growth under illumination was slightly decreased over that measured with 5 μ M TAPP alone, with the combination also showing an $\sim$ 1–2 log decrease over control levels which was statistically significant. It is worth noting that at sub-MIC levels, TAPP inhibits bacterial growth more effectively than sub-MIC levels of ceftriaxone or vancomycin alone; together they provide a 2–3 log decrease in bacterial growth. Interestingly, addition of 5 μ M TAPP to 4.5 mg/L tobramycin (Figure 3c) or 2 mg/L chloramphenicol (Figure 3d, protein synthesis inhibitors) resulted in an additional $\sim$ 2.5 or 3 log inhibition of bacterial growth over that observed with 4.5 mg/L tobramycin or 2 mg/L chloramphenicol. These decreases were not only significantly different than control values but also showed a statistically significant decrease over the values observed with 0.5X MIC photoactivated TAPP alone ($p < 0.05$ for all). When compared to 5 μ M TAPP (0.5X MIC), these combinations resulted in $\sim$ 3 (tobramycin + TAPP) and $\sim$ 2 (chloramphenicol + TAPP) log decreases in bacterial growth.

3.4 TAPP + antibiotics: additive or synergistic?

Because TAPP combined with tobramycin or chloramphenicol caused marked decreases in numbers of viable bacteria, we asked if these combinations were additive or synergistic. In this breakpoint-like assay, additivity or synergism occurs when bacterial growth is inhibited beyond that which would be measured with the individual drug itself (Figure 3a and Figure 3b). FICI values of 1.0 and 0.56 were measured for TAPP in combination with tobramycin and chloramphenicol, respectively, indicating that the combined TAPP + antimicrobials have additive effects on *S. aureus* (Figure 4). Against *E. coli*, these combinations still showed additivity, albeit the additivity was minimal. Importantly, against *S. epidermidis* and a clinical strain of *MRSA*, these two antibiotics are synergistic (Figure 4c). Because the dye methylene blue also releases ROS during illumination [25, 26], we performed analogous experiments using a similar photo-activated checkerboard method. Using chloramphenicol+ methylene blue, similar additive effects were measured with *S. aureus* (FICI range=0.375–1.0).

3.5 TAPP cytotoxicity

The cytotoxicity of TAPP was measured using Saos-2 osteosarcoma cells. In the dark, no significant differences between 0 – 100 μ M TAPP were observed by Trypan blue exclusion (Figure 6ai-iv). After illumination for 5 h, cells exposed to 1, 10 and 100 μ M TAPP showed marked increases in the number of trypan blue-positive cells (Figure 6av-viii). When blue cells were counted, there were no significant differences between control and TAPP-treated samples in the dark (Figure 6b). In the light, control samples (0 mM TAPP) had 6 ± 3 stained cells/FOV, while there were 81 ± 38 , 81 ± 41 , and 222 ± 80 cells/FOV stained with trypan blue for 1, 10, and 100 μ M TAPP, respectively (Figure 6c).

4. DISCUSSION

In this manuscript, we hypothesized that a photosensitizing agent could act in combination with waning concentrations of antibiotics to maintain antimicrobial prophylaxis. Here we have shown that TAPP, a *meso*-substituted cationic porphyrin possesses antimicrobial activity and is phototoxic towards multiple strains of bacteria. We have shown that photo-activated TAPP exhibits a dose- and time-dependent toxicity towards bacteria that can be partially abrogated by the antioxidant glutathione suggesting that the effects, at least partially, are due to the production of ROS in the presence of light. Importantly, we have examined the ability of TAPP to act in concert with two classes of antibiotics, those that target the cell wall (vancomycin and ceftriaxone) and those that target protein synthesis

(tobramycin and chloramphenicol). The membrane active antibiotics, vancomycin and ceftriaxone show only modest effects when combined with TAPP. The protein synthesis inhibitors, tobramycin and chloramphenicol show additive effects for *S. aureus* and *E. coli*. Interestingly, using MRSA and *S. epidermidis*, TAPP added to tobramycin or chloramphenicol displays synergy, even when sub-MIC levels of TAPP and antibiotics are used, suggesting that the combination can provide a more potent activity. Finally, we have shown that TAPP also exhibits a dose-dependent eukaryotic cell toxicity when TAPP is photoactivated.

TAPP, a cationic porphyrin shows a time-dependent and dose-dependent antibacterial activity against the Gram positive *S. aureus*, with a MIC of 10 μM a value consistent with MICs of other porphyrins of its class [27–29]. Similarly, *S. aureus* was sensitive to methylene blue. Yu et al. reported that while cationic porphyrins are active against Gram positive bacteria, Gram negative bacteria were resistant [30]. Still, when combined with additives such as detergents that increase membrane permeability [31], phototherapy could be used against Gram negative bacteria. Interestingly, we found that the Gram negative bacteria *E. coli* was susceptible to TAPP (MIC $\sim 60 \mu\text{M}$, or roughly six times the MIC of TAPP for *S. aureus* ($\sim 10 \mu\text{M}$)), whereas the *E. coli* was resistant to the effects of methylene blue. The relative resistance of Gram-negative bacteria to photosensitizers is thought to arise as the outer envelope of Gram negative species protects against efficient binding/internalization of the photoactivated compounds and/or the Gram-negative bacteria are less susceptible to the effects of reactive oxygen species [10]. It is interesting that this cationic porphyrin TAPP still has toxicity, albeit reduced, towards the Gram negative bacteria *E. coli*. Importantly, we do not see any change in sensitivity to TAPP or to other antibiotics throughout these experiments. These results are in accordance with previous experiments for other porphyrins conducted by Mendes et al [32].

Because others have attributed the antibacterial effects of activated porphyrins to their ability to produce ROS, especially in the *meso*-substituted porphyrin family [27–29] of which TAPP is a member, we included the antioxidant glutathione to scavenge ROS during the photo-activation of TAPP and thus protect against oxidative damage to bacteria [33]. It is worth noting that TAPP tends to age/aggregate over time, especially in the presence of bacterial media, causing spatially heterogeneous ROS production. As ROS is labile and has a mean free path of $\sim 0.4 \mu\text{m}$ [31], such aggregation could cause uneven effects on glutathione and on bacteria. To ensure equivalence between experiments as well as monitor any aging of solutions over time, TAPP MICs were routinely determined and the TAPP solution was discarded if the MIC had increased by $>20\%$. Glutathione decreased but did not eliminate the effects of photo-activated TAPP. It is probable that at least at 1 h, ROS generation exceeds the radical scavenging capacity of glutathione. However, changes in membrane potential, respiration, and membrane permeability accompany use of photoactivated porphyrins, suggesting an integrated effect of ROS with other bacterial functions [34]. As glutathione was only partially successful in abrogating the antibacterial effects of photoactivated TAPP, we would suggest that some of these other effects of porphyrin on bacterial function are also crucial for the action of TAPP.

Because the effects of this photo-activation are necessarily short-lived, we asked if repeated illuminations of TAPP would continue to have anti-microbial effects. Each round of illumination decreased the characteristic peak absorbance of porphyrin and also the antibacterial activity. However, even after 5 rounds of illumination, sufficient intact TAPP remained to decrease bacterial growth by two logs at 5 h. The ability to re-activate this mixture increases the utility of this agent. Similar advantages have been seen with other porphyrins, which may be repeatedly used as light-activated catalysts until they are either modified or completely degraded [35].

We hypothesized that TAPP would be a good candidate for use in combination antimicrobial therapies. As TAPP targets bacterial membranes, we reasoned that antibiotics that targeted protein synthesis would be more likely to complement TAPP activity than membrane-active antibiotics which could share sites of action. In fact, when combined with TAPP, the antimicrobials chloramphenicol and tobramycin showed additive activity against *S. aureus* and *E. coli*; against *S. epidermidis* and a clinical *MRSA* strain they showed synergy. In contrast, the membrane-active antibiotics, ceftriaxone and vancomycin only modestly increased activity of TAPP alone. This synergy is especially interesting for *MRSA* as we measured the MIC of TAPP for *MRSA* at 32.5 μM indicating diminished sensitivity to photodynamic therapy; antibiotic sensitivity of *MRSA* may be normal or attenuated for antibiotics such as ciprofloxacin and tobramycin. As FICI measures a ratio of activities, it is possible that the boost that the photodynamic therapy gives to antibiotic action is sufficient to overcome some of the innate resistance. Others have shown that effects of methylene blue are at least additive with tobramycin, meropenem, ceftriaxone, and chloramphenicol when tested against *B. cepacia* biofilms. [13]. Studies that used the porphyrin TMP with *P. aeruginosa* [14] or *S. aureus* [15] used the agents sequentially so that combination effects could not be determined. The finding of synergy for these strains is suggestive that combined use of these agents may be a powerful tool for combating infections.

Finally, we explored the toxicity of porphyrins to eukaryotic cells, specifically the immortalized osteosarcoma cell line Saos-2. Rapidly multiplying cells may be more prone to incorporation of porphyrins in the cell membrane, resulting in increased cell death when the porphyrin is photo-activated. In fact, porphyrins are commonly used as a form of photodynamic therapy for tumor destruction and neoplasia [9, 36]. Maisch, et al. have reported that two photo-active porphyrins did not influence cell viability without illumination, however with 5 min illumination, eukaryotic cells were significantly less viable at concentrations starting at 10 μM [37]. With TAPP, even with 5 h of illumination, significant numbers of cells survived which is in contrast with Maisch's report where phototoxicity after 24 h was greater than 50% for two porphyrins at 1 and 10 μM (5 min illumination). Consistent with Maisch's results, TAPP cytotoxicity at higher concentrations was significant, limiting the levels of photosensitizers that can be used in these therapies. Importantly, the effects reported for this cell line are likely to represent stringent toxicity measurements from cells particularly sensitive to TAPP exposure.

The data presented in this manuscript supports the utility of combination therapies with the cationic porphyrin TAPP and antibiotics that have inhibit protein synthesis; we would suggest that the antibiotics that target DNA replication would also show at least additivity. It is interesting to note that bactericidal antibiotics cause a prolonged production of ROS during bacterial killing [38], but the role of ROS in antibiotic activity remains questionable [39, 40]. Thus, the role of ROS in the combined activities, i.e., beyond that already noted for the porphyrins, is questionable. Overall, the proposed addition of photosensitizers to antibiotics may allow increased activity against a range of clinically significant pathogens, including *MRSA*, and act to supplement waning concentrations of antibiotics to maintain efficacy.

Acknowledgments

Our thanks to Professor Irving Shapiro, Professor Donald Jungkind and Brian Pepe-Mooney for many helpful discussions. Ms. Dastgheyb's work was supported by the NIH (National Institute of Arthritis and Musculoskeletal and Skin Diseases) training grant T32-AR-052273. The authors thank the NIH R01HL060230 (D.M.E), HD061053 and DE019901 (N.J.H.), as well as the NSF Nano-Bio Interface Center DMR08-32802 (R.J.C. and N.J.H.) for support of this work.

References

1. Thompson RL, Cabezudo I, Wenzel RP. Epidemiology of nosocomial infections caused by methicillin-resistant *Staphylococcus aureus*. *Ann Internal Med*. 1982; 97:309–317. [PubMed: 7114627]
2. Liu C, Bayer A, Cosgrove S, Daum R, Fridkin S, Gorwitz R, Kaplan S, Karchmer A, Levine D, Murray B, Rybak M, Talan D, Chambers H. Clinical practice guidelines by the infectious diseases society of America for the treatment of methicillin-resistant *Staphylococcus aureus* infections in adults and children. *Clin Infect Dis*. 2011; 52:285–292. [PubMed: 21217178]
3. Lowy FD. Antimicrobial resistance: the example of *Staphylococcus aureus*. *J Clin Invest*. 2003; 111:1265–1273. [PubMed: 12727914]
4. Lowy FD. *Staphylococcus aureus* infections. *New Engl J Med*. 1998; 339:520–532. [PubMed: 9709046]
5. Hamblin M, Hasan T. Photodynamic therapy: a new antimicrobial approach to infectious disease? *Photochem Photobiol Sci*. 2004; 3:436–450. [PubMed: 15122361]
6. Biesaga M, Pyrzy ska K, Trojanowicz M. Porphyrins in analytical chemistry. A review. *Talanta*. 2000; 51:209–224. [PubMed: 18967853]
7. Derosa MC, Crutchley RJ. Photosensitized singlet oxygen and its applications. *Coord Chem Rev*. 2002; 234:351–371.
8. Xing C, Xu Q, Tang H, Liu L, Wang S. Conjugated polymer/porphyrin complexes for efficient energy transfer and improving light-activated antibacterial activity. *J Am Chem Soc*. 2009; 131:13117–13124. [PubMed: 19702260]
9. Komagoe K, Kato H, Inoue T, Katsu T. Continuous real-time monitoring of cationic porphyrin-induced photodynamic inactivation of bacterial membrane functions using electrochemical sensors. *Photochem Photobiol Sci*. 2011; 10:1181–1188. [PubMed: 21472187]
10. Reddi E, Ceccon M, Valduga G, Jori G, Bommer J, Elisei F, Latterini L, Mazzucato U. Photochemical properties and antibacterial activity of *meso*-substituted cationic porphyrins. *Photochem Photobiol*. 2002; 75:462–470. [PubMed: 12017471]
11. Moan J. Porphyrin-sensitized photodynamic inactivation of cells: A review. *Lasers Med Sci*. 1986; 1:5–12.
12. Kolarova H, Nevrellova P, Tomankova K, Kolar P, Bajgar R, Mosinger J. Production of reactive oxygen species after photodynamic therapy by porphyrin sensitizers. *Gen Physiol Biophys*. 2008; 27:101–105. [PubMed: 18645224]
13. Cassidy CM, Donnelly RF, Elborn JS, Magee ND, Tunney MM. Photodynamic Antimicrobial Chemotherapy (PACT) in combination with antibiotics for treatment of *Burkholderia cepacia* complex infection. *J Photochem Photobiol B*. 2012; 106:95–100. [PubMed: 22079165]
14. Collins TL, Markus EA, Hassett DJ, Robinson JB. The effect of a cationic porphyrin on *Pseudomonas aeruginosa* biofilms. *Curr Microbiol*. 2010; 61:411–416. [PubMed: 20372908]
15. Di Poto A, Sbarra MS, Provenza G, Visai L, Speziale P. The effect of photodynamic treatment combined with antibiotic action or host defence mechanisms on *Staphylococcus aureus* biofilms. *Biomaterials*. 2009; 30:3158–3166. [PubMed: 19329182]
16. Cahan R, Schwartz R, Langzam Y, Nitzan Y. Light-activated antibacterial surfaces comprise photosensitizers. *Photochem Photobiol*. 2011; 87:1379–1386. [PubMed: 21883241]
17. White RL, Burgess DS, Manduru M, Bosso JA. Comparison of three different *in vitro* methods of detecting synergy: time-kill, checkerboard, and E test. *Antimicrob Agents Ch*. 1996; 40:1914–1918.
18. Sabbahi S, Alouini Z, Jemli M, Boudabbous A. The role of reactive oxygen species in *Staphylococcus aureus* photoinactivation by methylene blue. *Water Sci Technol*. 2008; 58:1047–1054. [PubMed: 18824803]
19. Watts JS, Thomas Apleay, Michael, Bade Donald J, Brown Steven D, Gray Jeffrey T, Heine Henry, Hunter Rob P, Mevius Dik J, Papich Mark G, Silley Peter, Zurenko GE. Approved Standard — Third Edition. 28., Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals: Approved Standard. *Clin Lab Standards Inst*. 2008; 28:M31–A33.

20. Sabath L, Garner C, Wilcox C, Finland M. Susceptibility of *Staphylococcus aureus* and *Staphylococcus epidermidis* to 65 antibiotics. *Antimicrob Agents Ch.* 1976; 9:962–969.
21. McLaughlin J, Murray PR. In vitro activity of ceftriaxone and other cephalosporins against 602 clinical isolates of *Staphylococci* from geographically diverse medical centers. *Eur J Epidemiol.* 1993; 9:685–661.
22. Tenover F, Biddle J, Lancaster M. Increasing resistance to vancomycin and other glycopeptides in *Staphylococcus aureus*. *Emerging infectious diseases.* 2001; 7:327–332. [PubMed: 11294734]
23. Lorian, V. Antibiotics in laboratory medicine. 5. Lippincott Williams & Wilkins; Philadelphia: 2005.
24. Lu N, Chen W, Peng YY. Effects of glutathione, Trolox and desferrioxamine on hemoglobin-induced protein oxidative damage: anti-oxidant or pro-oxidant? *Eur J Pharmacol.* 2011; 659:95–101. [PubMed: 21419762]
25. Ibrahim AM, Al-Ashqar SM. Spectroscopic and kinetic studies on the degradation of methylene blue using the supramolecular coordination polymer [(Ph₃Sn)₄FeCN(6)] as catalyst. *Spectrochim Acta A.* 2012; 92:238–244.
26. McCaughan B, Rouanet C, Fowley C, Nomikou N, McHale AP, McCarron PA, Callan JF. Enhanced ROS production and cell death through combined photo- and sono-activation of conventional photosensitising drugs. *Bioorg Med Chem Lett.* 2011; 21:5750–5752. [PubMed: 21875807]
27. Alves E, Costa L, Carvalho CM, Tome JP, Faustino MA, Neves MG, Tome AC, Cavaleiro JA, Cunha A, Almeida A. Charge effect on the photoinactivation of Gram-negative and Gram-positive bacteria by cationic *meso*-substituted porphyrins. *BMC Microbiol.* 2009; 9:70. [PubMed: 19368706]
28. Burda WN, Fields KB, Gill JB, Burt R, Shepherd M, Zhang XP, Shaw LN. Neutral metallated and *meso*-substituted porphyrins as antimicrobial agents against Gram-positive pathogens. *Eur J Clin Microbiol Infect Dis.* 2012; 31:327–335. [PubMed: 21667268]
29. Reddi E, Ceccon M, Valduga G, Jori G, Bommer JC, Elisei F, Latterini L, Mazzucato U. Photophysical properties and antibacterial activity of *meso*-substituted cationic porphyrins. *Photochem Photobiol.* 2002; 75:462–470. [PubMed: 12017471]
30. Yu J, Ye J, Liu X, Han Y, Wang C. Protective effect of L-carnitine against H₂O₂-induced neurotoxicity in neuroblastoma (SH-SY5Y) cells. *Neurol Res.* 2011; 33:708–716. [PubMed: 21756550]
31. Merchat M, Bertolini G, Giacomini P, Villanueva a, Jori G. *Meso*-substituted cationic porphyrins as efficient photosensitizers of Gram positive and Gram negative bacteria. *J Photochem Photobiol B.* 1996; 32:153–157. [PubMed: 8622178]
32. Mendes S, Camacho F, Silva T, Calado C, Serra A, Gonsalves A, Roxo-Rosa M. A nonionic porphyrin as a noninterfering DNA antibacterial agent. *Photochem Photobiol.* 2011; 87:1395–1404. [PubMed: 21834867]
33. He YY, Häder D. UV-B-induced formation of reactive oxygen species and oxidative damage of the cyanobacterium *Anabaena sp.*: protective effects of ascorbic acid and N-acetyl-L-cysteine. *J Photochem Photobiol B.* 2002; 66:115–124. [PubMed: 11897511]
34. Kato H, Komagoe K, Inoue T, Katsu T. In situ monitoring of photodynamic inactivation of the membrane functions of bacteria using electrochemical sensors. *Anal Sci.* 2010; 26:1019–1021. [PubMed: 20953042]
35. Bonnett R, Martinez G. Photobleaching of sensitizers used in photodynamic therapy. *Tetrahedron.* 2001; 57:9513–9547.
36. Takasaki A, Aoki A, Mizutani K, Wang SFSAC, Koshy G, Romanos G, Izumi Y II. Application of antimicrobial photodynamic therapy in periodontal and peri-implant diseases. *Periodontol* 2000. 2009; 51:109–140. [PubMed: 19878472]
37. Maisch T, Bosl C, Szeimies RM, Lehn N, Abels C. Photodynamic Effects of Novel XF Porphyrin Derivatives on Prokaryotic and Eukaryotic Cells Photodynamic Effects of Novel XF Porphyrin Derivatives on Prokaryotic and Eukaryotic Cells. *Antimicrob Agents Ch.* 2005; 49:1542–1552.
38. Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ. A common mechanism of cellular death induced by bactericidal antibiotics. *Cell.* 2007; 130:797–810. [PubMed: 17803904]

39. Keren I, Wu Y, Inocencio J, Mulcahy LR, Lewis K. Killing by bactericidal antibiotics does not depend on reactive oxygen species. *Science*. 2013; 339:1213–1216. [PubMed: 23471410]
40. Liu Y, Imlay JA. Cell death from antibiotics without the involvement of reactive oxygen species. *Science*. 2013; 339:1210–1213. [PubMed: 23471409]
41. Kuimova M, Botchway S, Parker A, Balaz M, Collins H, Anderson H, Suhling K, Ogilby P. Imaging intracellular viscosity of a single cell during photoinduced cell death. *Nature Chemistry*. 2009; 1:69–73.

Highlights

- Meso-Tetra (4-aminophenyl) Porphine (TAPP) is a light, dose, and time-dependent antimicrobial
- Antibiotics + illuminated TAPP are at least additive for *S. aureus*, *S. epidermidis*, MRSA, and *E. coli*
- TAPP can be used for several cycles before losing phototoxicity through photodegradation

Figure 1. Porphyrin properties and experimental setup

a. Structure of the *meso*-substituted porphyrin, TAPP. **b.** Schematic outline of photosensitized singlet oxygen production (adapted from [41]); irradiation of the photosensitizer, TAPP, results in a singlet excited state, S_1 ; intersystem crossing from the S_1 state results in the triplet state, T_1 ; toxic singlet oxygen is generated from ground state oxygen via energy transfer as T_1 returns to ground state. **c.** experimental setup for TAPP activation in solution under irradiation. **d.** absorption spectrum of 30 μM TAPP in H_2O after 0 (Fresh), 1, 5, or 10-rounds of 5 h light/19 h dark.

Figure 2. Under illumination, TAPP inhibits *S. aureus* growth

a. Numbers of viable *S. aureus* after 5-h incubation in 10 or 100 μM TAPP in either light or dark conditions. **b.** Viable *S. aureus* numbers in the presence of 0 μM (Control), 0.5 μM (0.5X MIC) or 50 μM (5X MIC) TAPP for 1–5 h under illumination. **c.** Bacterial proliferation in the presence of 20 μM TAPP + 0, 5, 10, or 20 mM glutathione (GSH), an antioxidant, during photoactivation. **d.** *S. aureus* proliferation in the presence of 10 μM TAPP during illumination for 1–5 h (fresh) or in the presence of 10 μM TAPP that had undergone a 5 h illumination/18–24 h dark cycle as indicated. Controls measure *S. aureus* proliferation during illumination without TAPP. For all experiments, n = 3 for each data point. Experiments were repeated 3 times with similar results. * indicates significance using a one-way ANOVA as compared with control, p < 0.05.

Figure 3. Sub-MIC levels of photo-activated TAPP increase antimicrobial activity in combination with sub-MIC levels of antibiotics

Dotted line represents initial inoculum. **a.** Ceftriaxone. **b.** Vancomycin. **c.** Tobramycin. **d.** Chloramphenicol. All conditions were illuminated during the 5 h test and retrieved *S. aureus* plated and counted. $n=3$ for each condition in each experiment. Experiments were repeated 3 times with similar results. * indicates significance using a one-way ANOVA as compared with control, $p < 0.05$.

Figure 4. Checkerboard assays

a. *S. aureus* growth (top: 96 well plate with cartoon of wells showing bacterial population below it) in the presence of tobramycin (0–32 mg/L) + TAPP (0–20 μM). **b.** *S. aureus* growth (top: 96 well plate with cartoon of wells showing bacterial population below it) in the presence of chloramphenicol (0–32 mg/L) + TAPP (0–20 μM). **c.** Table showing calculated FICI range of checkerboard results for *S. aureus*, *E. coli*, *S. epidermidis*, and a clinical *MRSA* isolate using TAPP with either tobramycin or chloramphenicol.

Figure 5. Photo-activated TAPP and eukaryotic cell toxicity

a. Representative images of control and TAPP treated cells stained with Trypan Blue after 5 h of incubation. Magnification: scale bar=200 μ m. **b.** Enumeration of cells/field of view (FOV) stained with Trypan Blue for cells incubated in the dark $p=0.08$ (control vs. 1 μ M); 0.31 (control vs. 10 μ M); 0.071 (control vs. 100 μ M) (**b**) or light (**c**) for 5 h; *= significant difference where $p=0.020$ (control vs. 1 μ M); 0.022 (control vs. 10 μ M); 0.0046 (control vs. 100 μ M).