Amphiphilic tetracationic porphyrins are exceptionally active antimicrobial photosensitizers: In vitro and in vivo studies with the free-base and Pd-chelate

Weijun Xuan1,2,3 | Liyi Huang2,3,4 | Yuguang Wang2,5 | Xiaoqing Hu2,6 | Grzegorz Szewczyk7 | Ying-Ying Huang2,3 | Ahmed El-Hussein2,8 | Jerry C. Bommer9 | Mark L. Nelson9 | Tadeusz Sarna7 | Michael R. Hamblin2,3,10*

1Department of Otorhinolaryngology, Head and Neck Surgery, First Clinical Medical College and Hospital, Guangxi University of Chinese Medicine, Nanning, China
2Wellman Center for Photomedicine, Massachusetts General Hospital, Boston, Massachusetts
3Department of Dermatology, Harvard Medical School, Boston, Massachusetts
4Department of Infectious Diseases, First Affiliated Hospital, Guangxi Medical University, Nanning, China
5Center of Digital Dentistry, Peking University School and Hospital of Stomatology, Beijing, China
6State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi, China
7Department of Biophysics, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland
8The National Institute of Laser Enhanced Science, Cairo University, Giza, Egypt
9Frontier Scientific, Inc., Logan, Utah
10Harvard-MIT Division of Health Sciences and Technology, Cambridge, Massachusetts

*Correspondence
Michael R. Hamblin, Harvard-MIT Division of Health Sciences and Technology, Cambridge, MA 02139.
Email: hamblin@helix.mgh.harvard.edu

Abstract
Antimicrobial photodynamic inactivation (aPDI) employs the combination of nontoxic photosensitizing dyes and visible light to kill pathogenic microorganisms regardless of drug-resistance, and can be used to treat localized infections. A meso-substituted tetra-methylpyridinium porphyrin with one methyl group replaced by a C12 alkyl chain (FS111) and its Pd-derivative (FS111-Pd) were synthesized and tested as broad-spectrum antimicrobial photosensitizers when excited by blue light (5 or 10 J/cm²). Both compounds showed unprecedented activity, with the superior FS111-Pd giving 3 logs of killing at 1 nM, and eradication at 10 nM for Gram-positive methicillin-resistant Staphylococcus aureus. For the Gram-negative Escherichia coli, both compounds produced eradication at 100 nM, while against the fungal yeast Candida albicans, both compounds produced eradication at 500 nM. Both compounds could be categorized as generators of singlet oxygen (ΦΔ = 0.62 for FS111 and 0.71 for FS111-Pd). An in vivo study was carried out using a mouse model of localized infection in a partial thickness skin abrasion caused by bioluminescent Gram-negative uropathogenic Escherichia coli. Both compounds were effective in reducing bioluminescent signal in a dose-dependent manner when excited by blue light (405 nm), but aPDI with FS111-Pd was somewhat superior both during light and in preventing recurrence during the 6 days following PDT.

KEYWORDS
amphiphilic tetracationic porphyrins, antimicrobial photodynamic inactivation, bioluminescence imaging, broad-spectrum activity, palladium-porphyrin, uropathogenic Escherichia coli infection
INTRODUCTION

The alarming rise of multidrug resistance (MDR) amongst pathogenic bacteria caused consternation in 2015 when the O’Neill report was published [1]. This report predicted that by 2050 (if nothing were done to stem the growth of MDR bacteria) there would have been 300 million premature deaths that would have cost the world economy $100 trillion. Fears of the emergence of untreatable infections have led to calls for alternative nonantibiotic approaches to be developed to alleviate the coming catastrophe [2, 3]. One of the most promising and innovative approaches to kill drug-resistant bacteria and treat resistant infections is known as antimicrobial photodynamic inactivation (aPDI). aPDI uses nontoxic photosensitizers (PS) that are excited by visible light in the presence of oxygen, and go on to produce singlet oxygen and other cytotoxic reactive oxygen species (ROS) by photochemical reactions.

A large number of PS possessing a variety of structures have been designed to target and kill various classes of microorganisms including bacteria, fungi, viruses and parasites [4, 5]. It is remarkable that there has been a wide range of activity reported between all these different structures with the most active being able to kill Gram-positive bacteria in the low nanomolar range [6], while the least active may need concentrations as high as low mM (6 orders of magnitude difference).

A previous publication [7] described the preparation and photophysical properties of a series of tetracationic porphyrins based on a meso-substituted tetrapyridinium porphyrin, 5,10,15,20-tetrakis-(4-N-methylpyridyl) porphyrin (T4MPyP), where one N-methyl group is replaced by a hydrocarbon group of 12 carbons in length, are synthesized depicted in Scheme 1. 21H,23H-Porphine, 5,10,15,20-tetra-4-pyridinyl (I), was prepared using standard methods [9]. One gram (1.61 mmoles) 21H,23H-Porphine, 5,10,15,20-tetra-4-pyridinyl (I) plus 290 mg (1.29 mmoles) of 1-bromododecane were refluxed for 22 hours in 20 mL of glacial acetic acid. Thin layer chromatography on C18 silica reversed-phase plates eluting with 90/10 methanol/6 N HCl v/v showed approximately 35% to 40% of the mono-N-dodecyl derivative, 1-dodecyl-4-(10,15,20-tri-4-pyridinyl-21H,23H-porpholin-5-yl) pyridinium (II), with slightly more starting porphyrin and a trace (approximately 2%) of the di-N-dodecyl derivative (<2%) and the solvent was removed by rotary evaporation.

In the present study, we describe the preparation of an analogous porphyrin (FS111) with an intermediate length alkyl chain (C12). Moreover, we also prepared the palladium central-substituted analog (FS111-Pd) [7, 8]. These compounds were tested for aPDI against Gram-positive and Gram-negative bacteria and fungal cells in vitro. We also tested the ability of these compounds to mediate aPDT of a localized Gram-negative bacterial infection, caused by uropathogenic E. coli in a partial thickness abrasion wound model in mice.

METHODS

2.1 Synthesis and characterization of porphyrins

Derivatives of meso-tetra-(N-methyl-pyridyl) porphyrin, having four quaternized nitrogens, where one N-methyl group is replaced by a hydrocarbon group of 12 carbons in length, are synthesized depicted in Scheme 1. 21H,23H-Porphine, 5,10,15,20-tetra-4-pyridinyl (I), was prepared using standard methods [9]. One gram (1.61 mmoles) 21H,23H-Porphine, 5,10,15,20-tetra-4-pyridinyl (I) plus 290 mg (1.29 mmoles) of 1-bromododecane were refluxed for 22 hours in 20 mL of glacial acetic acid. Thin layer chromatography on C18 silica reversed-phase plates eluting with 90/10 methanol/6 N HCl v/v showed approximately 35% to 40% of the mono-N-dodecyl derivative, 1-dodecyl-4-(10,15,20-tri-4-pyridinyl-21H,23H-porpholin-5-yl) pyridinium (II), with slightly more starting porphyrin and a trace (approximately 2%) of the di-N-dodecyl derivative (decreasing Rf with increasing substitutions).

The mixture was poured into 200 mL of methanol and cooled to 4°C, to precipitate the starting porphyrin. The precipitate was collected by centrifugation, and the decanted solvent containing the product was dried via rotary evaporation. The resulting solid was reflushed in 150 mL of methanol for 4 hours, cooled to 4°C, and the resulting solid was removed by centrifugation. Thin-layer chromatography of the decanted solution (same system as above) showed predominantly the mono-dodecyl substituted product with di-N-dodecyl derivative (<2%) and the solvent was removed by rotary evaporation. The solid obtained was further purified using C18-reverse phase preparative high-pressure liquid

Foundation, Grant/Award Numbers:
2014GXNSFAA118162, 2016GXNSFAA380312; Guangxi Scientific and Technological Project, Grant/Award Number: 1355005-1-2; National Natural Science Foundation of China, Grant/Award Numbers: 81774374, 81373700, 81260552, 8160369, 81472002, 81260239; National Institutes of Health, Grant/Award Numbers: R21AI121700, R01AI050875

INTRODUCTION

The alarming rise of multidrug resistance (MDR) amongst pathogenic bacteria caused consternation in 2015 when the O’Neill report was published [1]. This report predicted that by 2050 (if nothing were done to stem the growth of MDR bacteria) there would have been 300 million premature deaths that would have cost the world economy $100 trillion. Fears of the emergence of untreatable infections have led to calls for alternative nonantibiotic approaches to be developed to alleviate the coming catastrophe [2, 3]. One of the most promising and innovative approaches to kill drug-resistant bacteria and treat resistant infections is known as antimicrobial photodynamic inactivation (aPDI). aPDI uses nontoxic photosensitizers (PS) that are excited by visible light in the presence of oxygen, and go on to produce singlet oxygen and other cytotoxic reactive oxygen species (ROS) by photochemical reactions.

A large number of PS possessing a variety of structures have been designed to target and kill various classes of microorganisms including bacteria, fungi, viruses and parasites [4, 5]. It is remarkable that there has been a wide range of activity reported between all these different structures with the most active being able to kill Gram-positive bacteria in the low nanomolar range [6], while the least active may need concentrations as high as low mM (6 orders of magnitude difference).

A previous publication [7] described the preparation and photophysical properties of a series of tetracationic porphyrins based on a meso-substituted tetrapyridinium porphyrin, 5,10,15,20-tetrakis-(4-N-methylpyridyl)-porphine (T4MPyP), where one N-methyl group was replaced by a hydrocarbon group of 12 carbons in length, are synthesized depicted in Scheme 1. 21H,23H-Porphine, 5,10,15,20-tetra-4-pyridinyl (I), was prepared using standard methods [9]. One gram (1.61 mmoles) 21H,23H-Porphine, 5,10,15,20-tetra-4-pyridinyl (I) plus 290 mg (1.29 mmoles) of 1-bromododecane were refluxed for 22 hours in 20 mL of glacial acetic acid. Thin layer chromatography on C18 silica reversed-phase plates eluting with 90/10 methanol/6 N HCl v/v showed approximately 35% to 40% of the mono-N-dodecyl derivative, 1-dodecyl-4-(10,15,20-tri-4-pyridinyl-21H,23H-porpholin-5-yl) pyridinium (II), with slightly more starting porphyrin and a trace (approximately 2%) of the di-N-dodecyl derivative (decreasing Rf with increasing substitutions).

In the present study, we describe the preparation of an analogous porphyrin (FS111) with an intermediate length alkyl chain (C12). Moreover, we also prepared the palladium central-substituted analog (FS111-Pd) [7, 8]. These compounds were tested for aPDI against Gram-positive and Gram-negative bacteria and fungal cells in vitro. We also tested the ability of these compounds to mediate aPDT of a localized Gram-negative bacterial infection, caused by uropathogenic E. coli in a partial thickness abrasion wound model in mice.

METHODS

2.1 Synthesis and characterization of porphyrins

Derivatives of meso-tetra-(N-methyl-pyridyl) porphyrin, having four quaternized nitrogens, where one N-methyl group is replaced by a hydrocarbon group of 12 carbons in length, are synthesized depicted in Scheme 1. 21H,23H-Porphine, 5,10,15,20-tetra-4-pyridinyl (I), was prepared using standard methods [9]. One gram (1.61 mmoles) 21H,23H-Porphine, 5,10,15,20-tetra-4-pyridinyl (I) plus 290 mg (1.29 mmoles) of 1-bromododecane were refluxed for 22 hours in 20 mL of glacial acetic acid. Thin layer chromatography on C18 silica reversed-phase plates eluting with 90/10 methanol/6 N HCl v/v showed approximately 35% to 40% of the mono-N-dodecyl derivative, 1-dodecyl-4-(10,15,20-tri-4-pyridinyl-21H,23H-porpholin-5-yl) pyridinium (II), with slightly more starting porphyrin and a trace (approximately 2%) of the di-N-dodecyl derivative (decreasing Rf with increasing substitutions).

The mixture was poured into 200 mL of methanol and cooled to 4°C, to precipitate the starting porphyrin. The precipitate was collected by centrifugation, and the decanted solvent containing the product was dried via rotary evaporation. The resulting solid was reflushed in 150 mL of methanol for 4 hours, cooled to 4°C, and the resulting solid was removed by centrifugation. Thin-layer chromatography of the decanted solution (same system as above) showed predominantly the mono-dodecyl substituted product with di-N-dodecyl derivative (<2%) and the solvent was removed by rotary evaporation. The solid obtained was further purified using C18-reverse phase preparative high-pressure liquid
chromatography (HPLC) (Phenomenex Luna C18 10 μm, 100 A, 21.5 x 150 mm custom packed column) following a binary gradient system at 30 mL/minute and detection at 254 nm where Solvent A = 0.1% trifluoroacetic acid (TFA) and Solvent B acetonitrile (ACN) with 0.1% TFA. Rt = 22.3 to 22.5 minutes, where fractions 5 to 12 were pooled and the solvent removed in vacuo to produce the product, 1-dodecyl-4-(10,15,20-tri-4-pyridinyl-21H,23H-porphin-5-yl) pyridinium (II), in >98% purity as determined by HPLC (Phenomenex Kinetix column, 2.6 μm C18 100 A., 50 x 4.6 mm column) using a binary gradient system of Solvent A = 0.1% TFA, Solvent B = ACN with 0.1% TFA with detection at 254 nm and a 0 to 100% gradient (A to B) over 10 minutes. Rt = 6.39 minutes. Proton (1H) NMR were performed on a Varian 400 MHz instrument in DMSO-d6 and expressed in δ in ppm: -3.0 (2H), 0.95 (t, 3H), 1.2 to 1.7 (br, m, 18 H), 2.2 to 2.4 (m, 2H), 4.9 to 5.0 (m, 2H), 8.55 to 8.65 (m, 6H), 9.0 to 9.4 (m, 16H), 9.6 to 9.65 (m, 2H). High Resolution Mass Spectrometry (HRMS) was performed (Triclinic Laboratories, Lafayette, IN) using a Bruker AutoFlex III MALDI-TOF/TOF instrument with a Smartbeam Nd:YAG laser and using a matrix of α-cyano-4-hydroxycinnamic acid. Chemical Formula: C_{52}H_{51}N_{8}, Calculated mass (m/z) HRMS = 787.4200, Found m/z HRMS = 787.6610.

The solid II was dissolved in 50 mL of N,N-dimethylformamide containing 1.0 gram of methyl-p-toluenesulfonate, and refluxed for 2 hours. The solvent was removed by rotary evaporation, and the solid dissolved in 50 mL of deionized water and filtered to remove insoluble material. The solution was applied to an 8 cm x 2 cm chromatography column packed with 40 μm butyl (C4) reversed phase packing (J. T. Baker). The column was washed briefly with 3 N HCl, then eluted under isocratic conditions with 50% methanol, 50% 1 N HCl. The main product, 4,4',4''-[20-(1-dodecylpyridinium-4-yl)-21H,23H-porphine-5,10,15-triyl]tris[1-methyl] pyridinium tetrachloride (III), designated FS111), was collected and dried by rotary evaporation to yield 400 mg of a shiny dark green powder which was estimated to be greater than 95% pure by thin layer chromatography on Whatman KC2 silica reversed-phase plates eluting with 50% methanol and 50% 1 N HCl v/v Rf = 0.2. The retention time by HPLC was performed using an Altex Ultrasphere silica column and a binary gradient system at 1.5 mL/min. Solvent A = 1 M KNO₃ Solvent B = 90% ACN at a gradient of 0% A to 30% over 20 minutes. UV absorbance was detected at 419 nm. Rt = 11.70 minutes with 99.0% purity by AUC. Proton (1H) NMR were performed on a JEOL 400 MHz instrument in DMSO-d6 and expressed in δ in ppm: −3.1 (s, 2H), 0.85 (t, 3H), 1.25 to 1.70 (m, 18H), 2.25 to 2.40 (m, 2H), 4.75 (s, 9H), 4.8 to 4.85 (m, 2H), 5.0 to 5.1 (m, 2H), 8.9 to 9.0 (m, 8H), 9.2 to 9.4 (m, 8H), 9.2 to 9.4 (m, 8H). High Resolution Mass Spectrometry MALDI TOF (HRMS) was performed using a matrix of α-cyano-4-hydroxycinnamic acid. Chemical Formula: C_{55}H_{60}N_{8}, Calculated mass (m/z) HRMS = 832.4900, Found m/z HRMS = 834.6320.
the compound, palladium 4′,4″-[20-(1-dodecylpyridinium-4-yl)-21H,23H-porphine-5,10,15-triyl]tris[1-methyl]pyridinium tetrachloride (IV), in 89% yield. The retention time by HPLC was performed using an Altex Ultrasphere silica column and a binary gradient system at 1.5 mL/min. Solvent A = 1 M KNO₃ Solvent B = 90% acetonitrile at a gradient of 0% A to 60% 15 mins. UV absorbance detected at 419 nm. Retention time = 11.53 mins >95% purity by AUC. Proton (¹H) NMR were performed on a JEOL 400 MHz instrument in DMSO-d₆ and expressed in ppm: 0.9 (t, 3H), 1.25 to 1.75 (br m, 18 H), 2.3 to 2.5 (m, 2H), 4.80 (s, 9H), 5.0 to 5.1 (m, 2H), 8.9 to 9.0 (m, 8 H), 9.1 to 9.2 (m, 8H), 9.4 to 9.5 (m, 8H) [9–12]. High Resolution Mass Spectrometry MALDI TOF (HRMS) was performed using a matrix of α-cyano-4-hydroxycinnamic acid. Chemical Formula: C₅₅H₅₈N₈Pd, Calculated mass (m/z) = 936.3800, Found HRMS = 935.4240.

2.2 | Optical absorption and emission spectroscopy

Absorbance and fluorescence emission measurements were carried out using 1-cm-optical path quartz fluorescence cuvette (QA-1000; Hellma, Mullheim, Germany). For absorbance measurements, samples of FS111 and FS111Pd dissolved in water were scanned in the spectral range 200 to 900 nm using HP 8453 spectrophotometer (Hewlett-Packard, Palo Alto, California). Emission spectra were collected using samples prepared for absorption measurements, except the concentration of samples was reduced so the absorbance at 420 nm did not exceed 0.4. The emission measurements were carried out using LS55 luminospectrometer (Perkin-Elmer Inc., Waltham, Massachusetts). Samples were excited at 420 nm, and the detected emission was recorded between 430 and 900 nm. Both excitation and emission monochromators slits were adjusted to 4 nm.

2.3 | Time-resolved singlet oxygen detection

Phosphate-buffered (pH 7.2) D₂O solutions of FS111 or FS111Pd in a 1-cm-optical path quartz fluorescence cuvette (QA-1000; Hellma, Mullheim, Germany) were excited by pulses generated by an integrated nanosecond DSS Nd:YAG laser system equipped with a narrow bandwidth optical parametric oscillator (NT242-1 k-SF/SFG; Ekspla, Vilnius, Lithuania), which delivered pulses at repetition rate 1 kHz, with energy up to several hundred microjoules in the visible region. To adjust photoexcitation energy, laser beam was attenuated with up to six pieces of wire mesh (light transmission of each piece ~35%). To determine quantum yield of singlet oxygen photogeneration by the porphyrins, (4-N,N,N, N-trimethylaminium)porphyrin (TAP) was used as a reference, with the determined quantum yield 0.67. Quantum yields of singlet oxygen photogeneration by the porphyrins were determined by comparative measurements of the initial intensities of 1270-nm phosphorescence emitted by TAP and the porphyrins excited with 422-nm laser pulses of increasing energies. The concentration of TAP and the studied porphyrins was adjusted such that their absorbances at 422 nm were 0.148. The near-infrared luminescence was measured perpendicularly to the excitation beam in a photon-counting mode using a thermoelectric cooled NIR PMT module (H10330-45; Hamamatsu, Japan) equipped with a 1100-nm cutoff filter and an additional dichroic narrow-band filter NBP, selectable from the spectral range 1150 to 1355 nm (NDC Infrared Engineering Ltd, Bates Road, Maldon, Essex, UK). Data were collected using a computer-mounted PCI-board multichannel scaler (NanoHarp 250; PicoQuant GmbH, Berlin, Germany). Data analysis, including first-order luminescence decay fitted by the Levenberg-Marquardt algorithm, was performed by custom-written software. Acquisition time for obtaining melamin action spectra was 10 seconds.

2.4 | Electron paramagnetic resonance spin trapping studies

EPR spin trapping was employed using DMPO as a spin trap. EPR quartz flat cell, filled with solution of 0.025 mM FS111 or FS111Pd in 90% DMSO and 10% H₂O, and 100 mM DMPO, was irradiated in the resonant cavity with blue light (402-508 nm, 24 mW/cm²) derived from a 300-W high pressure compact arc xenon lamp (Cermax, PE300CE-13FM/Module300W; PerkinElmer Opto-electronics, GmbH, Wiesbaden, Germany) equipped with a water filter, heat reflecting hot mirror, cutoff filter blocking light below 390 nm and blue additive dichroic filter 505FD64-25 (Andover Corporation, Salem, North Carolina). EPR samples were run using microwave power 10.6 mW, modulation amplitude 0.05 mT, center field 339.0 mT, scan width 4 mT, and scan time 21 seconds, employing Bruker EMX-AA EPR spectrometer (Bruker BioSpin, Rheinstetten, Germany).

2.5 | Microbiology

The following strains were employed. Gram-positive bacterium, methicillin-resistant Staphylococcus aureus (MRSA) USA300, Gram-negative bacteria, E. coli K-12 (ATCC 33780), fungal yeast, luciferase-expressing Candida albicans strain (CEC 749). A colony of bacteria or C. albicans was routinely grown in 20 mL of brain heart infusion (BHI) broth (Fishier Scientific, Braintree, Massachusetts) for bacteria, or yeast extract-peptone-dextrose (YPD) broth (Sigma-Aldrich, St. Louis, Missouri) for C. albicans and grown overnight in a shaker incubator (New Brunswick
Scientific, Edison, NJ) at 120 rpm under aerobic conditions at 37°C for bacteria or at 30°C for \textit{C. albicans}. An aliquot of 1 mL from an overnight bacterial suspension was sub-cultured in fresh BHI for 2 to 3 hours at 37°C to mid-log phase. Cell concentration was estimated by measuring the optical density (OD) at 600 nm (OD of 0.6 = \(10^8\) CFU cells/mL). The \textit{C. albicans} cell number (\(10^7\) cells/mL) was assessed with a hemocytometer. The suspension of \textit{C. albicans} (\(10^7\) cells/mL) or bacteria (\(10^8\) cells/mL) was centrifuged, washed with pH 7.4 phosphate-buffered saline (PBS), and resuspended in PBS at the cell density of \(10^7\) CFU/mL for \textit{C. albicans} or \(10^8\) cells/mL for bacteria.

\subsection*{2.6 In vitro aPDI studies}

Suspensions of bacteria (\(10^8\) cells/mL) or \textit{C. albicans} (\(10^7\) cells/mL) were incubated with various concentrations of FS111 or FS111-Pd in pH 7.4 PBS for 30 minutes in the dark at room temperature. An aliquot (200 \(\mu\)L) was transferred to a 96-well plate and illuminated with 5 or 10 J/cm\(^2\) of blue light (415 nm) from the top of the plates at room temperature. The light source we used was an Omnilux Clear-U light-emitting diode (LED) array (Photo Therapeutics, Inc., Carlsbad, California) that emitted blue light at a center wavelength of 415 nm to deliver 5 or 10 J/cm\(^2\) at an irradiance of 50 mW/cm\(^2\) as measured with a power meter (Coherent, Santa Clara, California). Another aliquot of 100 \(\mu\)L was used as the dark control (DC) from each sample. At the completion of illumination (or dark incubation), aliquots (100 \(\mu\)L) were taken from each well to determine CFU. As bacteria can settle at the bottom, care was taken to ensure that the contents of the wells were mixed thoroughly before sampling. The aliquots were tenfold serially diluted in PBS to give dilutions of \(10^{-1}\) to \(10^{-5}\) times in addition to the original concentration and 10 \(\mu\)L aliquots of each of the dilutions were streaked horizontally on square YPD agar plates for Candida or BHI agar plates for bacteria described by Jett et al [13]. Plates were incubated for 24 or 36 hours at 30°C (Candida) or for 12 to 18 hours at 37°C (bacteria) in the dark to allow colony formation. Each experiment was performed at least three times. A control group of cells treated with light alone (no FS111 or FS111-Pd 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 FS111 or FS111-Pd (or FS111 or FS111-Pd in the absence of light) to CFUs of microbes treated with neither.

\subsection*{2.7 Animal studies}

All animal experiments were approved by the IACUC of Massachusetts General Hospital and met National Institutes of Health (NIH) guidelines. Adult female BALB/c mice 6 to 8 week-old and weighing 18 to 21 g were used (Charles River Laboratories, Massachusetts). Mice were given access to food and water ad libitum, and maintained on a 12-hour light/dark cycle under a room temperature of 21°C. Group size was \(N = 5\) to 6 mice per group.

\subsection*{2.8 Mouse model of skin wound infection}

Mice were anesthetized by intraperitoneal (i.p.) injection of ketamine/xylazine cocktail. The dorsal skin of the mice was shaved by an electric razor. To create abrasion wounds, a surgical scalpel was used to gently scrape the epidermis off an area of approximately 1.0 cm X 1.0 cm area. The depth of the wound was no more than the shallow dermis. After creating the wounds, a 50 \(\mu\)L aliquot of bioluminescent bacterial suspension containing 5 X \(10^8\) CFU of \textit{E. coli} in sterile PBS was topically inoculated onto each defined area of the abrasion with a pipette tip. Bioluminescence images of the mice were captured by a luminescence camera immediately after the inoculation of bacteria to ensure that the bacterial inoculum applied to each abrasion was consistent. Buprenorphine (0.03 mg/kg of body weight subcutaneously twice a day) was injected to the mice for 3 days after the wound for pain relief.

\subsection*{2.9 Bioluminescence imaging}

The IVIS® Lumina Series III photon-counting camera (PerkinElmer, Inc., Waltham, Massachusetts) was used before, during, and after PDT and also daily after the creation of infection for 5 days. Photon counting mode was used and images were taken by detecting individual photons which were emitted by the bacterial cells without the need for any luciferase substrate. Mice were anesthetized by inhalation of an isoflurane/oxygen mixture for imaging. An adjustable stage was used to place the mice in the imaging chamber, the camera was just above the mouse. Gray-scale background image of each mouse was produced, followed by a bioluminescent image of the same area displayed in the red (strongest intensity) to blue (lowest intensity), and super-imposed on the gray-scale image. The bioluminescence image is quantized into the region of interest (ROI) using the absolute calibration data in the photon s-1 cm-2 sr-1 using IVIS software.

\subsection*{2.10 Mouse model of skin-abrasion infected \textit{E. coli} and follow-up}

Mice were randomly divided into 6 groups as follows: (A) Infected control group: abrasion wounds were only infected with \textit{E. coli} (\(n = 5\) mice); (B) FS111 Dark control
group: 50 μM FS111, no light (n = 5 mice); (C) FS111 PDT group: 50 μM FS111 irradiated with 415 nm light (n = 5 mice); (D) FS111-Pd Dark control group: 50 μM FS111-Pd, no light (n = 5 mice); (E) FS111-Pd PDT group: 50 μM FS111-Pd irradiated with 415 nm light (n = 6 mice); (F) 415 nm light-alone control group: irradiated with 415 nm blue light only, no PSs (n = 5 mice).

PS (FS111 or FS111-Pd) was added at 60 minutes after bacterial inoculation, after the infection had become established as confirmed by the bioluminescence imaging. The PS was added to the wounds as 50 μL of a PBS solution at a concentration of 50 μM and was allowed to incubate in the wound for 30 minutes in order to bind to and penetrate the bacteria. After 30 minutes the mice were imaged to quantify any dark toxicity of the PS to the bacteria. Blue light irradiation was delivered using a light-emitting diode (LED) (Vielight, Inc., Toronto, Canada) with peak emission at 415 nm and full-width half maximum of 20 nm. The LED was mounted on a heat sink to prevent thermal effects. The irradiance on the wound surface was 70 mW/cm² throughout the study as measured using a model DMM 199 power meter with 201 standard head (Coherent, Santa Clara, California). Cumulative doses of light of 0, 20, 40 and 80 J/cm² were delivered successively, accompanied by luminescence imaging and another addition 20 μL of the PS solution were performed after each aliquot of light dose. Sterile saline (0.5 mL intraperitoneally) was administered to support fluid balance during recovery. For (A), (B), (D) and (F) group, luminescence imaging and another addition 20 μL of the PS [For (B) and (D) group] or PBS [For (A) and (F) group] were performed at the same different time points as PDT group. To record the time course of the extent of bacterial infection, the bacterial bioluminescence from mouse wounds was measured daily for 5 days after the wounds until the infections were cured (characterized by the disappearance of bacterial luminescence) or the wounds were healed. Finally, the mice were euthanized.

3 | RESULTS

3.1 | Absorption and emission measurements

In water, at micromolar concentration, both porphyrin dyes exhibited absorption spectra indicating monomerization of the dye molecules (Figure 1A). Maximum absorbance in the Soret band is at 424 nm for FS111, and for FS111-Pd the maximum is shifted by 4 nm to shorter wavelength (420 nm). Weak Q-bands are observed in the spectral range 500 to 600 nm for FS111. In case of FS111-Pd, the corresponding Q-bands are reduced to two maxima, with the intensity two-fold higher than that of FS111. At 10-fold higher concentration (~0.015 mM), also the weakest Q-band of FS111 becomes detectable at ~635 nm (Figure 1A). On the other hand, under the conditions used FS111-Pd shows no detectable absorption at this wavelength.
When excited at 420 nm, the two porphyrin dyes exhibit in water very weak fluorescence emission (Figure 1B). The broad emission has a maximum around 660 nm and can be detected between 600 and 800 nm. The intensity of the fluorescence emission is about seven-fold stronger for FS111, compared to FS111Pd. The effect is probably due to shortening of the singlet excited state of the dye molecule induced by such a heavy metal as palladium.

3.2 | Photogeneration of singlet oxygen and superoxide anion

Both porphyrin dyes in phosphate-buffered D₂O exhibit significant formation of singlet oxygen. Data shown in Figure 2A indicate that the quantum yield of singlet oxygen photogeneration is 0.62 for FS111 and 0.71 for FS111-Pd, compared to 0.68 for TAP, which was determined by using rose Bengal as the primary standard. The results suggest that the chelation of Pd by the tetrapyrrrole ring in FS111-Pd has a small effect on the photophysics of the porphyrin and modifies only weakly its photochemical properties. Both porphyrin dyes photogenerated measurable fluxes of superoxide anion as indicated by the EPR-spin trapping measurements, using DMPO as a spin trap (Figure 2B). In air-saturated samples dissolved in 90% DMSO and 10% H₂O, the observed spectral features of the detected spin adduct are consistent with those of the DMPO-OOH, reported elsewhere [14, 15]. The rate of the spin adduct accumulation during irradiation of the porphyrins with blue light increases monotonically and is virtually the same for both dyes (Figure 2C). At longer irradiation times, such as 4.5 minutes in case of FS111 and about 6 minutes in case of FS111-Pd, the spin adduct signal intensity abruptly decreases, most likely due to one-electron reduction (photoreduction) of the spin adduct to a diamagnetic form.

3.3 | aPDI studies in vitro

Since it is known that Gram-positive MRSA is (in general) the easiest bacterial species to kill, we chose these cells for our first studies. We used blue light (415 ± 15 nm) to excite these porphyrins because the Soret band in the blue region of the absorption spectrum dominates the light absorption, compared to the much smaller Q-bands in the green and red regions.

Figure 3A shows the killing curves (with increasing porphyrin concentration) of Gram-positive MRSA obtained in conjunction with 0 J/cm² (dark), 5 J/cm² and 10 J/cm² 415 nm light. As the compounds were exceptionally active, we used concentrations of only 0.5, 1, 5 and 10 nM of FS-111 (free base). When 10 nM concentration was used, 3 or 4 logs of killing were obtained depending on the light dose. No dark toxicity was observed. Figure 3B shows the corresponding killing curves with FS-111-Pd. The Pd analog was even more active than the free base compound, with 10 J/cm² at 10 nM giving eradication (>6 logs of killing), and > 1 log of killing at the extremely low concentration of 1 nM.

Figure 4A,B shows the analogous killing curves obtained with the Gram-negative E. coli. As E. coli is known to be harder to kill than MRSA, we used 10 times higher (but still remarkably low) concentrations of 5, 10, 50 and 100 nM FS-111. Figure 4A shows only minor dark toxicity (<1 log of killing), but substantial light-mediated killing leading to eradication with only 100 nM and 10 J/cm², and 5 logs of killing with 5 J/cm². In Figure 4B, we see the corresponding curves with FS-111-Pd. Again the Pd compound was slightly more active than the free base with eradication obtained using only 50 nM and 10 J/cm².

As Candida is even harder to kill than E. coli, we increased the concentration even higher, and used 5, 10, 50, 100, 500 and 1000 nM concentrations of both compounds. Figure 5A shows the killing curves obtained with FS-111. There was no dark toxicity, but eradication (>5 logs
of killing) was obtained with 500 nM and 10 J/cm$^2$. In Figure 5B are shown the corresponding killing curves with FS-111-Pd, and again this compound was somewhat more effective than the free base, giving eradication with 500 nM and both light doses.

### 3.4 PDT of *E. coli* wound infection

We tested the in vivo ability of the two porphyrins to treat a real-life *E. coli* infection in a partial thickness skin abrasion. We employed a bioluminescent strain of *E. coli* that allowed us to use in vivo bioluminescence imaging to noninvasively follow the process of the infection in real time, both during the actual PDT procedure (by imaging the animals between successive doses of blue light), and in the days following PDT.

Figure 6A shows a panel of representative bioluminescence images obtained during the in vivo PDT procedure. The first column of images (bacteria) were captured immediately after addition of the bacteria to the wound. The second
column (bacteria +60 minutes) were captured after 1 hour to allow the bacteria to bind to the wound tissue. The third column (bacteria +60 minutes + 30 minutes PS) were after addition of the porphyrin and incubation (or incubation with PBS for control group and blue light group). Then images in groups 2, 4 and 6 were captured after delivery of 20 J/cm², 40 J/cm² and 80 J/cm² of 415 nm light. The only significant reductions were seen in the FS111 PDT group (group 4) and the FS111-Pd PDT group (group 6). The blue light alone showed only about 1 log of signal reduction at 80 J/cm²,

**FIGURE 5** aPDI of *C. albicans*. *C. albicans* (10⁷ cells/mL) incubated with increasing concentrations of porphyrins and excited by 0, 5, or 10 J/cm² of 415 nm light. (A) FS111; (B) FS111-Pd

**FIGURE 6** PDT in vivo of mice with *E. coli* wound infections. (A) Panel of representative bioluminescence images captured from mice undergoing PDT with FS111 or FS111-Pd and increasing fluences (20, 40 and 80 J/cm²) of 415 nm light. (B) Quantification of RLU values from bioluminescence images (n = 5-6 per group, bars SD)
while FS111-PDT showed 3 logs, and FS111-Pd PDT showed 4 logs (Figure 6B). The PDT effects of FS111-Pd + blue light was superior to that of FS111 + blue light and the difference approached statistical significance at 80 J/cm² ($P = 0.068$).

Figure 7A,B shows the results of the follow up studies. We monitored the mice for 5 days, starting the day after PDT by carrying out BLI every day. On day 1 post-PDT the control, FS111 dark, and FS111-Pd dark mice had substantial bioluminescence signal remaining. The blue light alone mice and the FS111 PDT mice had less than the former groups and had comparable bioluminescence values to each other, but the values from FS111-Pd-PDT treated mice were lowest of all the groups. The signal recurred significantly during the following days in the blue light group mice, while in the PDT groups the signal declined slightly. At days 2 to 3 the signal in FS111 PDT group was 10 times higher than the signal in the FS111-Pd PDT group, and in turn the signal in the blue light group was 10 times higher than the FS111 PDT signal. The signal in both the PDT groups had become undetectable by day 4, while there was still some signal remaining in the blue light group by day 5.

**FIGURE 7** Follow-up of mice treated with PDT of *E. coli* wound infections. (A) Panel of bioluminescence images captured from mice on day 1 (after PDT) up to the fifth day post-PDT with FS111 or FS111-Pd. (B) Quantification of RLU values from bioluminescence images ($n = 5-6$ per group, bars SD)

**4 | DISCUSSION**

These two cationic porphyrins were found to be exceptionally active as antimicrobial PS. While several different PS (cationic porphyrins and bacteriochlorins) have previously been found to be active against Gram-positive bacteria at concentrations in the nanomolar range (a few 10s to 100s of nM) [6, 16], the ability to eradicate bacteria with a concentration as low as 10 nM is unprecedented.

A series of porphyrins based upon the 5,10,15, 20-tetrakis-(4-N-methylpyridyl)-porphine (TMPyP4) backbone, where one N-methyl group was replaced by a hydrocarbon chain ranging from C6 through C10. C14, C18 to C22 was originally reported in 2002 [7]. Reddi et al compared the aPDI activity of these five porphyrins (and TMPyP4) against *S. aureus* and *E. coli* [7]. Using white light and porphyrin concentrations ranging from 400 nM to 8.3 μM they found that the C14 porphyrin was the most active, followed by the C10 analog and then the C18 analog. These results showed that there was an optimal length of the alkyl chain between C10 and C18 and the present study was undertaken to see if that optimal length might be C12.
A very preliminary study of the C12 compound (later to become known as FS111) was published in a book chapter in 2011 [8]. Therein, Jori et al briefly reported that the C12 porphyrin was actually more active than the C14 porphyrin in mediating aPDI against *S. aureus* and *E. coli*, thus giving us further encouragement to test FS-111.

It is well known that coordination of a central metal such as Pd into the tetrapyrole macrocycle increases the PS triplet yield due to the heavy atom effect [17, 18]. The inclusion of heavy atoms in the molecular structure increases the spin orbit coupling (SOC) between singlet and triplet states, which leads to more efficient inter-system crossing [19]. There are also many intriguing reports that Pd-coordinated bacteriochlorins and porphyrins may have a different photochemical mechanism, as compared to their free base counterparts (or even the zinc counterparts). In general, Pd-coordinated tetrapyroles appear to have a predominance of Type I photochemical mechanisms. This situation is exemplified by TOOKAD (a Pd-bacteriochlorophyll derivative) which has been reported to produce zero detectable singlet oxygen [20]. Mroz et al compared four imidazole porphyrins as photosensitizers for PDT killing of cancer cells, a free base, a Zn-chelate, an In-chelate, and a Pd-chelate [21]. They found that the Pd-porphyrin produced more hydroxyl radicals and was better at mediating PDT killing of cancer cells. The Zn-porphyrin produced the most singlet oxygen. Huang et al. [22] compared the PDT activity of three stable synthetic bacteriochlorins (a free base and Zn and Pd chelates). Again, the Pd-containing compound showed the highest phototoxicity towards cancer cells, and generated the most hydroxyl radicals. A subsequent molecular orbital study correlated the improved PDT activity of the Pd-containing dicyanobacteriochlorins and the propensity to carry out Type I photochemistry, with the redox potential [23]. In other words, the ability of the compounds to accept electrons (both in the singlet ground state and the excited triplet state) is higher for Pd-containing compounds and higher for Zn-containing compounds.

However, in the present case the Pd-chelate produced more singlet oxygen than the free base. Both compounds did produce superoxide at roughly the same rate. Although the redox potentials were not measured, it can be hypothesized that these porphyrins were not susceptible to being reduced, so the Pd atom made little difference in that regard, but the benefit from the heavy atom effect still applied.

Some factors that influence the structure-function relationships of antimicrobial photosensitizers are beginning to emerge [24]. It is well known that the presence of cationic charges is needed for activity against Gram-negative bacteria [4]. However, even though many cationic charges lead to higher activity against Gram-negative cells, too many cationic charges can be counter-productive against Gram-positive bacteria and fungi. We compared [25] four separate stable synthetic bacteriochlorins (BC) that had: (a) 2 basic nitrogens; (b) two quaternary ammonium groups; (c) four quaternary ammonium groups; (d) six quaternary ammonium groups against *S. aureus*, *E. coli* and *C. albicans*. Moreover, it is becoming increasingly clear that molecular asymmetry is also important. BC (a) was best at killing *Candida*, BC (b) was best at killing *S. aureus*, while BC (d) was best at killing *E. coli*. Hence, it appears that a large number of cationic charges is best for Gram-negative bacteria, while a modest number of cationic charges is optimum for the more porous Gram-positive bacteria, and no cationic charges (but a certain degree of basic character) are needed for the intermediate fungal yeast.

We studied [6] another set of stable synthetic bacteriochlorins (BC) that differed from the compounds described above, in that they were all monosubstituted, while the compounds described in [25] were all symmetrically disubstituted. These compounds had either one or two quaternary ammonium groups. The overall activity against every type of microbial cell tested (*S. aureus, E. coli, C. albicans*) was higher than might have been expected if the compounds had a symmetrical di-substituted structure with the same overall charge and hydrophobicity. The idea is that asymmetric amphiphilic compounds can insert themselves into lipid-bilayer membranes with the hydrophobic part of the molecule in the lipid membrane interior, and the cationic part in the aqueous exterior part.

It appears that another feature such as a carefully tailored degree of hydrophobicity is beneficial. The clear-cut evidence for this assertion comes from the present series of TMPyP4 derivatives [7, 8] where the C12 and C14 carbon chains showed superior activity compared to their close neighbors (C10 and C16 carbon chains).

The use of bioluminescence imaging to monitor in vivo PDT of infections in small animal models, has made the procedure considerably easier, faster, less expensive, and allows reduction of animal numbers [26]. In the present study, we chose to use a model of uropathogenic *E. coli* infection in partial thickness skin abrasions in mice. *Escherichia coli* possesses many different phenotypes that can cause a large variety of infections. Although many *E. coli* strains are considered to be normal intestinal flora, other strains are pathogenic and can cause serious food poisoning in humans. However, no single virulence gene including those encoding toxins, is the sine qua non of the potential of *E. coli* to cause disease [27]. *Escherichia coli* are also responsible for a majority of cases of urinary tract (UTIs) [28]. Uropathogenic *E. coli* (UPEC) is a member of what is known as “extraintestinal pathogenic *E. coli*” or ExPEC [29]. In addition to UTIs, ExPEC can cause neonatal meningitis, sepsis, pneumonia, surgical site and other wound infections [30].
Although blue light is much better than red light to excite porphyrins against planktonic cells in vitro, we wondered whether the same consideration applied to in vivo PDT. The bacteria take up residence in the burned skin tissue, and although these burns are relatively superficial infections, some depth of penetration of the light is still needed, and blue light may not be able to penetrate sufficiently. However, the palladium derivative had no detectable absorption in the red spectrum (see Figure 1A), so the use of red light was not possible. Blue light was relatively less effective in vivo than it had been in vivo, and a fluence of 80 J/cm² was necessary to obtain full eradication of the bioluminescence signal. However even 80 J/cm² of blue light is not exceptionally high when compared to the fluences of blue light that need to be used in vivo, when antimicrobial blue light alone studies are being considered [31]. Fluences as high as 432 J/cm² have been used [32]. Moreover, it was also interesting that bacterial regrowth occurring in the days following PDT was not as problematic as it has been in previous studies from our laboratory [33].

In conclusion, these two porphyrins (FS111 and FS111-Pd) show unprecedented activity as antimicrobial photosensitizers, and deserve further investigation.

ACKNOWLEDGMENTS

This work was supported by US NIH grants R01AI050875 and R21AI121700 (to M.R.H.). L.H. was supported by National Natural Science Foundation of China (81260239, 81472002, 81860369), Guangxi Scientific and Technological Project (1355005-1-2), Guangxi Natural Science Foundation (2016GXNSFAA380312). W.X. was supported by National Natural Science Foundation of China (81260552, 81373700, 81774374), Guangxi Natural Science Foundation (2014GXNSFAA118162). A.E.-H. was supported by The Fulbright Foundation. J.C.B. and M.L.N. are employees of Frontier Scientific, Inc., Logan, Utah. Research carried out at the Jagiellonian University (G.S. and T.S.) was supported in part by grants from the Poland National Science Center (2011/03/B/NZ1/00007 and 2013/08/W/NZ3/00700).

CONFLICT OF INTEREST

J.C.B. and M.L.N. are employees of Frontier Scientific, Inc., Logan, Utah, and declare a conflict of interest. M.R.H. declares the following potential conflicts. Dr Hamblin is on the following Scientific Advisory Boards, LLC, Cambridge, MA, Global Photon Inc., Bee Cave, TX, Medical Coherence, Boston MA, NeuroThera, Newark DE, JOOVV Inc., Minneapolis-St. Paul MN, AIRx Medical, Pleasanton CA, FIR Industries, Inc. Ramsey, NJ, UVLRx Therapeutics, Oldsmar, FL, Ultralux UV Inc., Lancing MI, Illumiheal & Petthera, Shoreline, WA, MB Lasertherapy, Houston, TX. Dr Hamblin has been a consultant for Lexington Int, Boca Raton, FL, USHIO Corp, Japan, Merck KGaA, Darmstadt, Germany, Philips Electronics Nederland B.V., Johnson & Johnson Inc., Philadelphia, PA, Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Germany. Dr Hamblin is a stockholder in Global Photon Inc., Bee Cave, TX, Mitonix, Newark, DE.

AUTHOR BIOGRAPHIES

Please see Supporting Information online.

ORCID

Michael R. Hamblin https://orcid.org/0000-0001-6431-4605

REFERENCES

[1] J. O’neill, Tackling a global health crisis: initial steps. in The Review on Antimicrobial Resistance Chaired by Jim O’Neill 2015. https://amr-review.org/sites/default/files/Report-52.15.pdf. Accessed April 22, 2019.
[2] A. Nigam, D. Gupta, A. Sharma, Microbiol. Res. 2014, 169 (9–10), 643.
[3] L. Czeplewski, R. Bax, M. Clokie, M. Dawson, H. Fairhead, V. A. Fischetti, S. Foster, B. F. Gilmore, R. E. W. Hancock, D. Harper, I. R. Henderson, K. Hilpert, B. V. Jones, A. Kadioglu, D. Knowles, S. Őlafsdóttir, D. Payne, S. Projan, S. Shaanak, J. Silverman, C. M. Thomas, T. J. Trust, P. Warn, J. H. Rex, Lancet Infect. Dis. 2016, 16(2), 239.
[4] S. K. Sharma, T. Dai, G. B. Kharkwal, et al., Curr. Pharm. Des. 2011, 17, 1303.
[5] R. Yin, M. R. Hamblin, Curr. Med. Chem. 2015, 22(18), 2159.
[6] L. Huang, M. Krayer, J. G. Roubil, et al., J. Photochem. Photobiol. B 2014, 141C, 119.
[7] E. Reddi, M. Ceccgon, G. Valduca, G. Jori, J. C. Bonmer, F. Elisei, L. Latterini, U. Mazzucott, Photochem. Photobiol. 2002, 75(5), 462.
[8] G. Jori, M. Camerin, M. Soncin, L. Guidolin, O. Coppellotti, Antimicrobial photodynamic therapy: basic principles. in Photodynamic Inactivation of Microbial Pathogens: Medical and Environmental Applications (Eds: M. R. Hamblin, G. Jori), RSC Publishing, Cambridge, UK 2011.
[9] S. Sugata, S. Yamanouchi, Y. Matsushima, Chem. Pharm. Bull. 1977, 25(5), 884.
[10] A. Antipas, M. Gouterman, J. Am. Chem. Soc. 1983, 105(15), 4896.
[11] J. C. Bonmer, G. Jori, Photodynamic porphyrin antimicrobial agents. US6573258 B2 2003.
[12] S. M. Borisov, I. Klimant, Dyes Pigments 2009, 83, 312.
[13] B. D. Jett, K. L. Hatter, M. M. Huycke, M. S. Gilmore, Biotechniques 1997, 23(4), 648.

[14] G. Szewczyk, A. Zadlo, M. Sarna, S. Ito, K. Wakamatsu, T. Sarna, Pigment Cell Melanoma Res. 2016, 29(6), 669.

[15] G. R. Buettner, R. P. Mason, Methods Enzymol. 1990, 186, 127.

[16] T. Maisch, C. Bosl, R. M. Szeimies, N. Lehn, C. Abels, Antimicrob. Agents Chemother. 2005, 49(4), 1542.

[17] G. R. Buettner, R. P. Mason, Methods Enzymol. 1990, 186, 127.

[18] T. Maisch, C. Bosl, R. M. Szeimies, N. Lehn, C. Abels, Antimicrob. Agents Chemother. 2005, 49(4), 1542.

[19] G. R. Buettner, R. P. Mason, Methods Enzymol. 1990, 186, 127.

[20] T. Maisch, C. Bosl, R. M. Szeimies, N. Lehn, C. Abels, Antimicrob. Agents Chemother. 2005, 49(4), 1542.

[21] G. R. Buettner, R. P. Mason, Methods Enzymol. 1990, 186, 127.

[22] T. Maisch, C. Bosl, R. M. Szeimies, N. Lehn, C. Abels, Antimicrob. Agents Chemother. 2005, 49(4), 1542.

[23] G. R. Buettner, R. P. Mason, Methods Enzymol. 1990, 186, 127.

[24] T. Maisch, C. Bosl, R. M. Szeimies, N. Lehn, C. Abels, Antimicrob. Agents Chemother. 2005, 49(4), 1542.

[25] G. R. Buettner, R. P. Mason, Methods Enzymol. 1990, 186, 127.

[26] T. Maisch, C. Bosl, R. M. Szeimies, N. Lehn, C. Abels, Antimicrob. Agents Chemother. 2005, 49(4), 1542.

[27] G. R. Buettner, R. P. Mason, Methods Enzymol. 1990, 186, 127.

[28] T. Maisch, C. Bosl, R. M. Szeimies, N. Lehn, C. Abels, Antimicrob. Agents Chemother. 2005, 49(4), 1542.